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The PEP–pyruvate–oxaloacetate node as the switch point for carbon flux distribution in bacteria

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We dedicate this paper to Rudolf K. Thauer, Director of the Max-Planck-Institute for Terrestrial Microbiology in Marburg, Germany, on the occasion of his 65th birthday

Abstract

In many organisms, metabolite interconversion at the phosphoenolpyruvate (PEP)–pyruvate–oxaloacetate node involves a structurally entangled set of reactions that interconnects the major pathways of carbon metabolism and thus, is responsible for the distribution of the carbon flux among catabolism, anabolism and energy supply of the cell. While sugar catabolism proceeds mainly via oxidative or non-oxidative decarboxylation of pyruvate to acetyl-CoA, anaplerosis and the initial steps of gluconeogenesis are accomplished by C3- (PEP- and/or pyruvate-) carboxylation and C4- (oxaloacetate- and/or malate-) decarboxylation, respectively. In contrast to the relatively uniform central metabolic pathways in bacteria, the set of enzymes at the PEP–pyruvate–oxaloacetate node represents a surprising diversity of reactions. Variable combinations are used in different bacteria and the question of the significance of all these reactions for growth and for biotechnological fermentation processes arises. This review summarizes what is known about the enzymes and the metabolic fluxes at the PEP–pyruvate–oxaloacetate node in bacteria, with a particular focus on the C3-carboxylation and C4-decarboxylation reactions in *Escherichia coli*, *Bacillus subtilis* and *Corynebacterium glutamicum*. We discuss the activities of the enzymes, their regulation and their specific contribution to growth under a given condition or to biotechnological metabolite production. The present knowledge unequivocally reveals the PEP–pyruvate–oxaloacetate nodes of bacteria to be a fascinating target of metabolic engineering in order to achieve optimized metabolite production.

Keywords: C3-carboxylation; C4-decarboxylation; Anaplerosis; Gluconeogenesis; Metabolic flux; PEP–pyruvate–oxaloacetate node

Contents

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1. Introduction

In most chemotrophic, aerobic and facultatively anaerobic bacteria, the Embden–Meyerhof–Parnas pathway (glycolysis) or the Entner–Doudoroff pathway and the tricarboxylic acid (TCA) cycle are the main pathways of central metabolism. The former two are the primary routes for carbohydrate breakdown to phosphoenolpyruvate (PEP), pyruvate and acetyl-CoA, thereby providing energy and building blocks for the synthesis of cellular components. The TCA cycle also serves a dual role in catabolism and anabolism by catalyzing complete oxidation of acetyl-CoA to CO_2 for respiratory ATP formation and by providing carbon precursor metabolites and NADPH for biosynthetic processes. Upon growth on TCA cycle intermediates or on substrates that enter central metabolism via acetyl-CoA (e.g. acetate, fatty acids and ethanol), the cycle intermediates malate or oxaloacetate must be converted to pyruvate and PEP for the synthesis of glycolytic intermediates. This gluconeogenic formation of sugar phosphates from PEP is accomplished by the reversible reactions of glycolysis and one further enzyme, fructose-1,6-bisphosphatase.

The metabolic link between glycolysis/gluconeogenesis and the TCA cycle is represented by the PEP–pyruvate–oxaloacetate node, also referred to as the anaplerotic node (Fig. 1). This node comprises a set of reactions that direct the carbon flux into appropriate directions and thus, it acts as a highly relevant switch point for carbon flux distribution within the central metabolism. Under glycolytic conditions, the final products of glycolysis PEP and pyruvate enter the TCA cycle via acetyl-CoA (oxidative pyruvate decarboxylation and fueling of the cycle) and via formation of oxaloacetate by carboxylation (C3-carboxylation). This latter route is referred to as anaplerosis, a process to replenish TCA cycle intermediates that were withdrawn for anabolic purposes [1–3]. Under gluconeogenic conditions, the TCA cycle intermediates oxaloacetate or malate are converted to pyruvate and PEP by decarboxylation (C4-decarboxylation) [1,4] and thus, the PEP–pyruvate–oxaloacetate node provides the direct precursors for gluconeogenesis.

Although essential, the carbon flux through the PEP–pyruvate–oxaloacetate node is flexible, and hence it is reasonable that the cells tightly adjust these fluxes to the energetic and anabolic demands under a given condition. In some bacteria and under some conditions, the regulation of the carbon flux at the PEP–pyruvate–oxaloacetate node is rather simple and straightforward. A prominent example is catabolite repression, which ensures absence of C4-decarboxylating enzymes at glucose

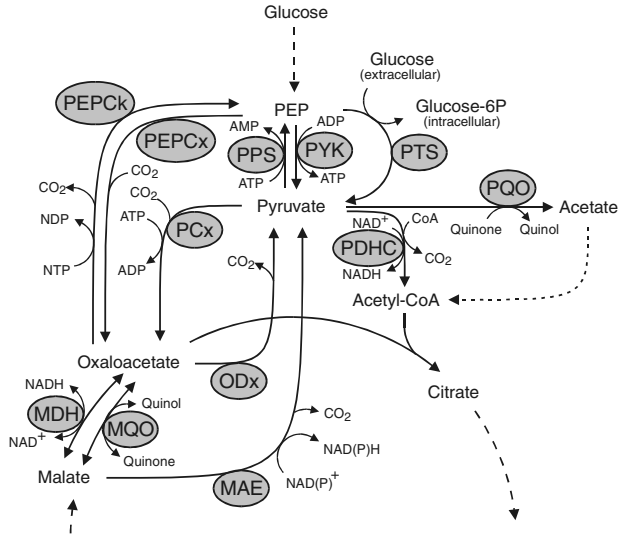

Fig. 1. The enzymes at the PEP–pyruvate–oxaloacetate node in aerobic bacteria. *Abbreviations:* MAE, malic enzyme; MDH, malate dehydrogenase; MQO, malate: quinone oxidoreductase; ODx, oxaloacetate decarboxylase; PCx, pyruvate carboxylase; PDHC, pyruvate dehydrogenase complex; PEPCk, PEP carboxykinase; PEPCx, PEP carboxylase; PPS, PEP synthetase; PQO, pyruvate: quinone oxidoreductase; PTS, phosphotransferase system; PYK, pyruvate kinase.

excess and presence during growth on gluconeogenic substrates [5–8]. Recent studies revealed, however, that the carbon flux control at the PEP–pyruvate–oxaloacetate node is often more complex than simple on/off regulation under a given condition. In some bacteria, two C3-carboxylating and up to three C4-decarboxylating enzymes are simultaneously active, even during growth on glucose as sole carbon and energy source (e.g. [9,10]). One enzyme, although operating in the same direction, can fulfil a different function under certain conditions (e.g. [11]). In other organisms or under different conditions, the same enzyme can operate in the reverse direction and thus contribute to a third function. These examples show that there is a complex interplay of the enzymes at the PEP–pyruvate–oxaloacetate node and it is obvious that sophisticated control is realized to ensure an optimal carbon and energy flow within central metabolism.

Based on biochemical, genetic and regulatory studies, on quantitative determinations of metabolic fluxes and on the genome sequences and expression profiling analyses, this paper summarizes what is known about the PEP–pyruvate–oxaloacetate node in different bacteria, with particular emphasis on the C3-carboxylation and C4-decarboxylation reactions in the model and industrial bacteria *Escherichia coli*, *Bacillus subtilis* and *Corynebacterium glutamicum*. We will discuss the organization and expression of genes, in vitro and in vivo enzyme activities, allosteric regulation, and the relevance of this central metabolic node for optimal growth and primary metabolite production under different conditions. It becomes evident that striking biochemical, genetic and regulatory peculiarities become important under certain conditions and the present knowledge unequivocally reveals the PEP–pyruvate–oxaloacetate nodes of bacteria to be fascinating targets of metabolic engineering in order to achieve optimized metabolite production.

2. The enzymes at the PEP–pyruvate–oxaloacetate node in bacteria

Fig. 1 summarizes pathways and enzymes that have been implicated in the PEP–pyruvate–oxaloacetate node of different bacteria. The figure gives an overview on what takes place in a hypothetical bacterial cell with features (enzyme equipments) of different species. Individual species contain typically only a subset of these reactions, with *C. glutamicum* as a prominent exception (Table 1). In particular the malic enzyme is often present as multiple isoenzymes, with varying cofactor specificities. Most aerobic microorganisms possess the pyruvate kinase and the pyruvate dehydrogenase complex that feed acetyl-CoA into the TCA cycle. Under anaerobic conditions, alternative enzymes for acetyl-CoA formation from pyruvate are pyruvate-formate lyase (in enterobacteria) and pyruvate-ferredoxin oxidoreductase (in saccharolytic clostridia) [12] (not shown in Fig. 1). Oxidative decarboxylation of pyruvate is also accomplished by the FAD-containing pyruvate oxidase (H_2O_2 and acetyl-phosphate-forming, in lactobacilli) [13–16] and pyruvate: quinone oxidoreductase (acetate-forming, in *E. coli* and *C. glutamicum*) ([17–19], M. Schreiner and B.J. Eikmanns, submitted for publication). Under fermentative conditions, pyruvate can be the substrate for pyruvate decarboxylase (e.g. in yeasts and in *Zymomonas mobilis*), lactate dehydrogenase (e.g. in lactic acid bacteria and enterobacteria) or acetolactate synthase (e.g. in *Enterobacter aerogenes*) (not shown in Fig. 1).

The anaplerotic function is accomplished in most bacteria by PEP and/or pyruvate carboxylase, which convert PEP or pyruvate, respectively, to oxaloacetate. Under gluconeogenic conditions, either PEP carboxykinase or oxaloacetate decarboxylase and/or malic enzyme in combination with PEP synthetase, serve for directing C4-intermediates from the TCA cycle to PEP, the direct precursor for gluconeogenesis. Table 1 gives an overview on the distribution of some of the enzymes at the PEP–pyruvate–oxaloacetate node in different bacteria. The situation is somewhat different in compartmentalized organisms such as the baker's yeast *Saccharomyces cerevisiae*, as was reviewed previously [20]. Here, pyruvate decarboxylase, the two pyruvate kinase isoenzymes, PEP carboxykinase, and the pyruvate carboxylase are located in the cytosol. In contrast to many higher organisms where the anaplerotic pyruvate carboxylase is a mitochondrial enzyme, its location is exclusively cytosolic in *S. cerevisiae* [20]. Since respiratory pyruvate catabolism is catalyzed by the mitochondrial TCA cycle, pyruvate is transported into the mitochondria and then converted by the pyruvate dehydrogenase complex. Similarly, the exclusive and NADP-dependent malic enzyme is located in the mitochondria [21].

In the following two sections, we will concentrate on the bacterial enzymes of the PEP–pyruvate–oxaloacetate node that direct the carbon flux either into anaplerosis or into gluconeogenesis, i.e. on the PEP-/pyruvate-carboxylating and oxaloacetate-/malate-decarboxylating enzymes.

2.1. C3-carboxylating enzymes

In principle, several enzymes are able to catalyze the carboxylation of PEP or pyruvate to oxaloacetate or malate, i.e. PEP carboxylase (EC 4.1.1.31; reaction (1)), PEP carboxykinase (EC 4.1.1.49 and EC 4.1.1.32; reaction (2)), PEP carboxytransphosphorylase (EC 4.1.1.38; reaction (3)), pyruvate carboxylase (EC

Table 1
Distribution of enzymes at the PEP–pyruvate–oxaloacetate node in different bacteria[a]

Organism	PEPCk[b]	PEPCx	PCx	ODx	MAE	PPS	PDHC
E. coli	1 (ATP)	1	0	0	1 (NAD) 1 (NADP) 1 (NADP)	1	1
C. glutamicum	1 (GTP)	1	1	1	1 (NADP)	0[c]	1
B. subtilis	1 (ATP)	0	1	0	2 (NAD) 1 (NADP)	0	1
Rhizobium etli [95]	1 (ATP)	1	1		1 (NAD) 1 (NADP)	1[d]	1
Sinorhizobium (Rhizobium) meliloti [95]	1 (ATP)	0	1		1 (NAD)[e]1 (NADP)[e]	1[d]	1
Rhodopseudomonas palustris [97]	1 (ATP)	1			1 (NAD)	1	1
Rhodobacter capsulatus [64]	1 (ATP)		1		1 (NAD)[f]		1
Ralstonia eutropha [83]	1 (GTP)			1	1 (NADP)	1	1
Thiobacillus novellus [287]		1	1		1 (NADP)		
Pseudomonas citronellolis [42]	0	1	1	1		1	1
Pseudomonas fluorescens [288–290]	1	1	1		1 (NADP)	1	1
Agrobacterium tumefaciens [291,292]	1	1	1		1 (NAD) 1 (NADP)	1	1
Arthrobacter globiformis [293]	1 (ATP)		1				1
Zymomonas mobilis [294]		1			1		1
Campylobacter jejuni [295]	1 (ATP)		1		1 (NAD)		

Please refer to the text for details on *E. coli*, *C. glutamicum*, and *B. subtilis*.

[a] the number gives the number of isoenzymes present in a given organism. Zero means that the organism has been tested for the enzyme or the respective gene, however, no activity has been found so far. Empty boxes (no number) mean that so far there is no evidence for the enzyme or a functional gene, however, the lack of evidence is no evidence of lack.

[b] For enzyme abbreviations see Fig. 1.

[c] In some *C. glutamicum* strains, PEP synthetase activity has been postulated by Jetten et al. [236] and by Vallino and Stephanopoulos [257], however, physiological studies [80] argue against a functional PEP synthetase in the wild-type strain *C. glutamicum* ATCC13032.

[d] Pyruvate orthophosphate dikinase.

[e] Unusually high MWs of 82 kDa that stem from a 320 C-terminal addition with similarity to *P*-acetyltransferases [296].

[f] [297].

6.4.1.1; reaction (4)) and malic enzyme (EC 1.1.1.40; reaction (5)). The PEP carboxykinase, PEP carboxytransphosphorylase and the malic enzyme reactions are regarded as reversible [2]:

$$\mathrm{PEP} + \mathrm{HCO_3^-} \rightarrow \mathrm{Oxaloacetate} + \mathrm{P}_i \quad (1)$$

$$\mathrm{PEP} + \mathrm{CO_2} + \mathrm{NDP} \rightleftarrows \mathrm{Oxaloacetate} + \mathrm{NTP} \quad (2)$$

$$\mathrm{PEP} + \mathrm{CO_2} + \mathrm{P}_i \rightleftarrows \mathrm{Oxaloacetate} + \mathrm{PP}_i \quad (3)$$

$$\mathrm{Pyruvate} + \mathrm{HCO_3^-} + \mathrm{ATP} \rightarrow \mathrm{Oxaloacetate} + \mathrm{ADP} + \mathrm{P}_i \quad (4)$$

$$\mathrm{Pyruvate} + \mathrm{HCO_3^-} + \mathrm{NAD(P)H} \rightleftarrows \mathrm{Malate} + \mathrm{NAD(P)} \quad (5)$$

Although all these enzymes are principally capable to catalyze the carboxylation reaction, the anaplerotic carboxylation function during growth of bacteria on carbohydrates is generally mediated by either PEP carboxylase, pyruvate carboxylase or PEP carboxytransphosphorylase [1–3,12,22]. The latter enzyme has so far been reported to be present only in propionic acid bacteria [2,23] and in *Acetobacterium woodii* [24], and very little is known about it. For these reasons, we confine our discussion on the C3-carboxylating enzymes on the PEP and pyruvate carboxylases and deal with the general aspects of PEP carboxykinase and malic enzyme in the following section.

PEP carboxylase was discovered in the fifties by Bandurski and Greiner [25] and since then has been found widely distributed in bacteria and plants. So far, it has never been observed in animals, yeast or fungi. The enzyme catalyzes the highly exergonic bicarbonate fixation on PEP to form oxaloacetate and inorganic phosphate, using Mg^{2+} or Mn^{2+} as a cofactor [1]. As was expected from the energetic point of view, the reaction is essentially irreversible and this has been shown by the fact that neither PEP formation from oxaloacetate nor exchange of $^{14}CO_2$ with oxaloacetate or $^{32}P_i$ exchange with PEP could be demonstrated [2].

PEP carboxylases have been purified from a wide range of bacterial sources and many biochemical data are available (e.g. [26–44]). With one exception (see next paragraph), all PEP carboxylases investigated so far are tetramers with subunit masses of between 90 and 110 kDa and based on biochemical experiments, amino acid alignments, and site-directed mutagenesis experiments, putatively important PEP carboxylase domains have been identified [45–47]. The three-dimensional structure of the PEP carboxylase from *E. coli* was analyzed by X-ray crystallographic studies [48,49] and conclusions

regarding the molecular reaction mechanism and the allosteric regulation of this enzyme were drawn and recently extensively reviewed by Kai et al. [50] and Izui et al. [51]. Most PEP carboxylases are sensitive to various metabolite effectors such as fatty acids, acetyl-CoA and fructose-1,6-bisphosphate as activators and aspartate and malate as allosteric inhibitors. In plants, PEP carboxylases additionally are subject to regulation by reversible phosporylation modification (reviewed in [52]), a process so far not detected in bacteria.

Very recently, a new type of PEP carboxylase (designated PEP carboxylase A) with no discernible evolutionary relationship to the hitherto known enzymes has been described for the archaeon *Methanothermobacter thermoautotrophicus* [53]. The subunit size of this homotetrameric enzyme is 55 kDa and in contrast to the well-known PEP carboxylases, its activity is not influenced by acetyl-CoA and it is much less sensitive to aspartate. According to the inspection of bacterial genomes, homologues of this PEP carboxylase are present in many archaea and possibly also in *Clostridium perfringens*, *Oenococcus oeni* and *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293A [53].

The PEP carboxylase genes (*ppc*) from several bacteria and also from a variety of plants have been cloned and the deduced proteins comparatively characterized (reviewed in [53,54]). The amino acid sequences of all eukaryotic and eubacterial PEP carboxylases are relatively well conserved, however, the plant PEP carboxylases clearly differ from the eubacterial enzymes and therefore, they are regarded as distinct evolutionary groups [47,54]. As indicated already above, the archaeal enzymes have a completely different amino acid sequences from all other eubacterial and plant PEP carboxylases, thus represent a distinct evolutionary branch.

Pyruvate carboxylase activity was for the first time described in chicken liver by Utter and Keech [55,56]. Generally, pyruvate carboxylases are biotin-containing enzymes and catalyze the irreversible carboxylation of pyruvate to oxaloacetate (reaction (4)). In contrast to PEP (the substrate of PEP carboxylase), pyruvate does not contain a high-energy phosphate bond and favourable thermodynamics for the carboxylation are achieved by ATP-dependent pyruvate carboxylase. The complete reaction consists of two steps, i.e. the ATP-dependent carboxylation of the enzyme-bound biotin (reaction (4a)) and the transfer of the activated carboxyl group onto pyruvate (reaction (4b)) [2,57–59].

$$\text{Enzyme–Biotin} + \text{ATP} + \text{HCO}_3^- \rightleftarrows \text{Enzyme–Biotin–COO}^- + \text{ADP} + \text{P}_i \quad (4a)$$

$$\text{Enzyme–Biotin–COO}^- + \text{pyruvate} \rightarrow \text{Enzyme–Biotin} + \text{oxaloacetate} \quad (4b)$$

For detailed and extensive overviews on the structure and function of pyruvate carboxylases from different sources (however, with special emphasis on eukaryotic sources) the reader should refer to recent reviews [59–61].

Whereas pyruvate carboxylase plays a major anaplerotic role in vertebrate tissues and in yeast, only few prokaryotes use it as the sole anaplerotic enzyme, e.g. the phototrophic *Rhodopseudomonas spheroides* [62] and *Rhodobacter capsulatus* [63,64] as well as *Arthrobacter globiformis* [65], *Mycobacterium smegmatis* [66], bacilli [67–70] and *Sinorhizobium meliloti* [71]. In many bacteria, such as *Pseudomonas citronellolis*, *P. fluorescens*, *Azotobacter vinelandii*, *Thiobacillus novellus*, *Rhizobium etli*, *M. thermoautotrophicum* and also in *C. glutamicum*, both pyruvate carboxylase and PEP carboxylase have been detected [36,40,42,72–75]. With the exception of *C. glutamicum* (for further details see Section 4), however, the relevance of the one or the other of the two enzymes under different growth conditions has not been clarified so far.

Pyruvate carboxylases from most species are activated by acetyl-CoA, but some bacterial enzymes (e.g. those of *Pseudomonas*, *M. thermoautotrophicum* and *C. glutamicum*) and also yeast pyruvate carboxylase show no or only slight dependence on the presence of acetyl-CoA [59,61]. With the exception of the *S. meliloti* pyruvate carboxylase, all bacterial pyruvate carboxylases tested are inhibited by aspartate and 2-oxoglutarate [63,71,76]. Furthermore, many bacterial pyruvate carboxylases are inhibited by AMP and ADP [63,73,77,78].

Native pyruvate carboxylases from most organisms represent tetramers of four identical subunits of about 120–130 kDa [61]. Some bacterial enzymes (e.g. those from *P. citronellolis*, *A. vinelandii* and *M. thermoautotrophicum*) have a different subunit structure and composition, i.e. they consist of two different subunits (α- and β subunit) forming a native $\alpha_4\beta_4$ enzyme [72,73,79]. The α subunit (about 65 kDa) carries the biotin moiety and contains the catalytically active sites for the reaction, the β subunit (about 55 kDa) is responsible for the conformational stability in the core of the enzyme [79]. Each subunit (or α subunit, respectively) contains tightly bound metal ions, i.e. Mn^{2+} and/or Mg^{2+} in most vertebrate enzymes, Mg^{2+} in bacterial enzymes and Zn^{2+} in case of the yeast pyruvate carboxylase [59].

The pyruvate carboxylase genes from a variety of bacteria have been isolated and characterized and highly conserved biotin carboxylation, transcarboxylation and biotin carboxyl carrier domains have been identified (e.g. [69,72,75,80]). Similar to the case with bacterial PEP carboxylase genes, transcriptional regulation of prokaryotic pyruvate carboxylase genes is not well studied. There are some indications for regulation at the genetic level by the observation of different specific activities in *A. vinelandii*, *R. shaeroides*, *R. capsulatus*

and *C. glutamicum* cells grown on different carbon sources, i.e. higher specific activities in lactate-grown cells than in glucose-, glycerol-, sucrose-, or malate-grown cells [62,64,73]. However, there are no experimental evidences for a transcriptional or translational regulation as the basis for these observations.

2.2. C4-decarboxylating enzymes

As mentioned in the previous section, PEP carboxykinase and malic enzyme catalyze reversible C3-carboxylation/C4-decarboxylation reactions. However, since in most bacteria these two enzymes are responsible for decarboxylation rather than for carboxylation reactions, they are treated in this section, together with the irreversible oxaloacetate decarboxylase (reaction (6)).

$$\text{Oxaloacetate} \rightarrow \text{Pyruvate} + CO_2 \quad (6)$$

PEP carboxykinase catalyzes the reversible decarboxylation and simultaneous nucleotide-dependent phosphorylation of oxaloacetate (reaction (2)) [1]. The enzyme is present in a variety of animals, plants, yeasts and microorganisms and depending on the nucleotide specificity, these enzymes can be divided into two classes [1]. The animal enzymes are exclusively monomeric and generally use GTP or ITP (EC 4.1.1.32). The plant, fungal and almost all bacterial enzymes, in contrast, are mono- or oligomeric and use ATP as the phosphate donor (EC 4.1.1.49) [81]. Exceptions from this rule are the PEP carboxykinases of *Ruminococcus flavefaciens* [82], *Ralstonia eutropha* [83], *A. globiformis* [1], *C. glutamicum* [84] and of the hyperthermophilic archaeon *Thermococcus kodakaraensis* [85], which have been shown to be GTP-dependent. However, as deduced from annotated (genome) sequences, further bacteria and archaea probably also possess GTP-specific PEP carboxykinases (summarized in [85]) and thus, it might turn out that in fact many if not most bacteria possess a GTP-dependent PEP carboxykinase. Although the overall amino acid sequence identity between the two classes of enzymes is very low (<20%), the ATP- and GTP-dependent PEP carboxykinases share conserved residues at the active site, i.e. nucleotide-, substrate- and metal-binding residues [81,85,86]. These findings corroborate the hypothesis that all PEP carboxykinases have retained the catalytically important motifs over a large evolutionary distance and probably operate with the same overall mechanism of catalysis [81]. Structure/function studies of the phosphoryl transfer by the *E. coli* PEP carboxykinase recently have been performed and extensively discussed by Delbaere et al. [87].

The physiological role of PEP carboxykinase depends on the species, on the presence or absence of other enzymes at the PEP–pyruvate–oxaloacetate node in a given organism and on the environmental conditions. In most organisms, the PEP carboxykinase reaction is the first step in gluconeogenesis, forming PEP from oxaloacetate [1]. While in some organisms this reaction is the only one converting C4-intermediates to PEP, many bacteria additionally possess oxaloacetate decarboxylase or malic enzyme and PEP synthetase (e.g. *R. palustris* or *E. coli*, see below) and thus, an alternative pathway from malate/oxaloacetate to PEP. A catabolic function of the C4-decarboxylation reaction by the PEP carboxykinase has recently been proposed for *E. coli* when growing slowly under glucose hunger in a continuous culture [11]. Here, PEP carboxykinase is one of the key enzymes in the so-called PEP–glyoxylate cycle (for details see Section 3.3). In few bacteria such as *R. eutropha* (and also in a pyruvate kinase-deficient mutant of *B. subtilis*, see Section 4.2), PEP carboxykinase has been found to catalyze the reverse reaction (i.e. C3-carboxylation) and thus to fulfil an anaplerotic function under glycolytic conditions [83,88]. In particular in rumen-, intestine-, and mouth-colonizing anaerobic bacteria such as *R. flavefaciens* and *Anaerobiospirillum succiniproducens* that require CO_2 to grow, PEP carboxykinase functions as a CO_2-fixing enzyme [82,89]. At least some PEP carboxykinases exhibit secondary activities as oxaloacetate decarboxylase or as pyruvate kinase [90], however, the physiological role of this side activities remains questionable.

A catabolic function of PEP carboxykinase has recently been also proposed for mammalian cells degrading amino acids [91]. Since the TCA cycle cannot fully oxidize C4- and C5-carbon compounds, which result from the degradation of amino acids, these C4- and C5-intermediates must be removed from the cycle. This is accomplished by a process known as cataplerosis and the initial reaction is that of PEP carboxykinase. Although proposed for mammalian cells, this function should also apply for bacteria when growing on a mixture of amino acids. In higher plants, the PEP carboxykinase recently also has been proposed to play a key role in amino acid metabolism, i.e. in the conversion of the carbon skeletons from the aspartate family of amino acids to those of glutamate/glutamine [92].

The expression of the PEP carboxykinase gene is controlled in different ways in different microorganisms. In most bacteria studied so far, expression of the respective *pckA* (or *pck*) gene is dependent on the carbon source in the growth medium, e.g. in *E. coli* [5,93,94], *S. meliloti* and other rhizobia [6,95], *Staphylococcus aureus* [8], *R. leguminosum* [7], *C. glutamicum* [84] and *T. kodakarensis* [85]. In all these organisms, PEP carboxykinase gene expression is low when they grow on glycolytic substrates and higher when they grow on gluconeogenic substrates. In *E. coli* and *S. meliloti*, the respective *pckA* gene additionally is strongly induced in the stationary growth phase [5,6,96], whereas in *R. palustris* it is strongly induced in the exponential growth phase, irrespective of the carbon source and under both anaerobic light and aerobic dark conditions [97]. Such a log-phase

induction has not been found for other *pckA* genes characterized to date. An explanation for the different regulation of the *pckA* gene in *R. palustris* might be that this organism can use sugar only poorly as a sole carbon source and therefore, PEP carboxykinase and gluconeogenesis is essential for the synthesis of carbohydrates and cell constituents in the exponential growth phase, irrespective of the carbon source.

Malic enzymes catalyze the reversible decarboxylation of malate to pyruvate with simultaneous reduction of primarily NADP (EC 1.1.140) or NAD (EC 1.1.1.38 and EC 1.1.1.39) (reaction (5)) [98]. One of the NAD-linked malic enzymes, i.e. EC 1.1.1.38, is also able to catalyze the decarboxylation of oxaloacetate, a property also shown for some of the NADP-linked but not shown for the other NAD-linked malic enzymes [98–102]. Most organisms possess an NADP-dependent malic enzyme, some bacteria such as *E. coli* and other enteric bacteria, *B. subtilis*, *Rhizobium* sp., *Pseudomonas* sp., *Alcaligines faecalis*, and *Agrobacterium tumefaciens* possess both (an) NAD- and an NADP-specific enzyme(s) (e.g. [60,67,103–106]). Lactic acid bacteria possess NAD-linked malic enzymes of the EC 1.1.1.39-type [107–110].

A function of either malic enzyme as CO_2-fixation system (C3-carboxylation) in bacteria has been excluded [3,111]. Instead it has become evident that in bacteria the NAD-dependent enzymes play a role in malate catabolism and that the NADP-dependent enzymes function as either gluconeogenic enzymes (supply of pyruvate from C4-dicarboxylic acids) or as NADPH generating system for biosynthetic purposes (Fig. 2) [3,98,112,113]. Moreover, as the PEP carboxykinase, the malic enzyme(s) may be responsible for the withdrawal of C4- and C5-intermediates from the TCA cycle and thus fulfil a catabolic function in cataplerosis (see above).

Malic enzymes from all types have been purified and biochemically characterized from a variety of bacteria, e.g. from *E. coli* (e.g. [102,114–116]), *Lactobacillus plantarum* [109], *C. glutamicum* [117], *B. stearothermophilus* [118], *P. fluorescens* [119], *S. meliloti* [60] *Bradyrhizobium japonicum* [120] and the archaeon *Sulfolobus solfataricus* [121]. In general, malic enzymes have a higher affinity towards malate than towards pyruvate, corroborating the C4-decarboxylation rather the C3-carboxylation function. Most of the characterized malic enzymes are allosterically controlled, i.e. positively and/or negatively regulated by a variety of metabolites of the central metabolism, and many of them are subject to substrate inhibition. The known biochemical and regulatory features of the malic enzymes from *E. coli*, *B. subtilis* and *C. glutamicum* are described in detail below.

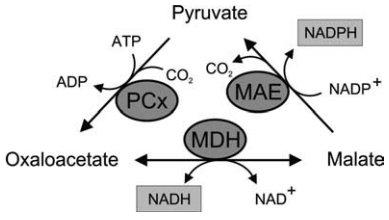

Fig. 2. NADPH formation from NADH in a cycle including the reactions of pyruvate carboxylase (PCx), malate dehydrogenase (MDH) and malic enzyme (MAE).

Oxaloacetate decarboxylase activity in a given organism may be due to activity of malic enzyme, pyruvate kinase, malate dehydrogenase, pyruvate carboxylase and PEP carboxykinase [101,122–124] or to the activity of "real" oxaloacetate decarboxylases (EC 4.1.1.3). These latter enzymes catalyze the irreversible decarboxylation of oxaloacetate [125] and can be classified into (i) the divalent cation-dependent oxaloacetate decarboxylases and (ii) the membrane-bound sodium-dependent and biotin-containing oxaloacetate decarboxylases from enterobacteria. This latter type of enzyme is involved in the fermentation of citrate by *Klebsiella pneumoniae* [42,126] and of citrate or D- or L-tartrate by *Salmonella typhimurium* [127,128] and is able to use the free energy of the C4-decarboxylation reaction to translocate sodium ions from the inside to the outside of the cell and thus to contribute to energy conservation. The structure, function, catalytic mechanism and regulation of the sodium-ion-translocating oxaloacetate decarboxylases were summarized in several recent reviews [126,129–134] and are therefore not discussed here. The former type of oxaloacetate decarboxylases are cytoplasmic enzymes, do not require sodium for activity, are not inhibited by avidin and are absolutely dependent on the presence of divalent cations such as Mn^{2+}, Co^{2+}, Mg^{2+}, Ni^{2+} or Ca^{2+}. They have been found in different microorganisms, e.g. in different species of *Pseudomonas* [42,135,136] and *Acetobacter* [137], *C. glutamicum* [138], *Veillonella parvula* [139] and *A. vinelandii* [140]. In some but not all of these organisms (e.g. in the pseudomonades), the oxaloacetate decarboxylase is inhibited by acetyl-CoA and ADP. A new type of Mn^{2+}-dependent oxaloacetate decarboxylase proposed to be involved in citrate fermentation of Gram-positive bacteria has recently been found in *Lactococcus lactis* [141]. As deduced from the respective *citM* gene, this enzyme shows a high level of similarity to malic enzymes from other organisms, however, at least in vitro, it has no malic enzyme activity.

The function of the divalent cation-dependent oxaloacetate decarboxylase in bacteria is not quite clear. It might have a function in gluconeogenesis in those organisms possessing a PEP synthetase. On the other side, the enzyme has been discussed to be essential for the maintenance of oxaloacetate during growth on glucose and for the supply of pyruvate during growth on intermediates of the TCA cycle [137].

3. The PEP–pyruvate–oxaloacetate node in *E. coli*

Despite its importance as the metabolic switch point in metabolism, the PEP–pyruvate–oxaloacetate node has been reviewed only tangentially in the unchallenged model bacterium *E. coli* with the primary focus on either glycolysis [142] or the TCA cycle [143]. Beyond a general review of the involved genes, proteins, and their regulation, we emphasize here the recent literature that approaches the overall function of the node in terms of deciphering in vivo molecular fluxes under varying conditions [11,144] and the relevance of flux redirections on the overall cell physiology [145,146]. This renewed interest in the PEP–pyruvate–oxaloacetate node was mostly driven by the need to manipulate carbon flux through the node in various biotechnological processes that will be discussed in Section 3.4. One particular problem in *E. coli* is the stoichiometric coupling of PEP conversion to two metabolic processes. Beyond the role as the phosphoryl donor for sugar uptake in the so-called PTS that is used by many bacteria [12], PEP is also the substrate for the PEP carboxylase-catalyzed anaplerotic reaction (Fig. 3). The essential nature of these processes reduce the availability of PEP as a building block for the production of, for example, aromatic amino acids and derived compounds [147,148].

3.1. *Anaplerosis in* E. coli

As a consequence of the anaplerotic configuration with PEP carboxylase as the exclusive C3-carboxylating enzyme, *E. coli* requires the *ppsA*-encoded PEP synthetase as an additional enzyme for growth on the C3-acids lactate, pyruvate, and alanine, which then contributes to both gluconeogenesis and anaplerosis (Fig. 3). To revert the highly exergonic pyruvate kinase reaction, PEP synthetase is coupled to ATP cleavage into AMP and PP_i, where the rapid cleavage of PP_i by pyrophosphorylase pulls the equilibrium far to the side of PEP synthesis [12]. The glyoxylate shunt constitutes a third anaplerotic reaction sequence that provides C4-compounds from the fusion of two C2-units during growth on acetate [12].

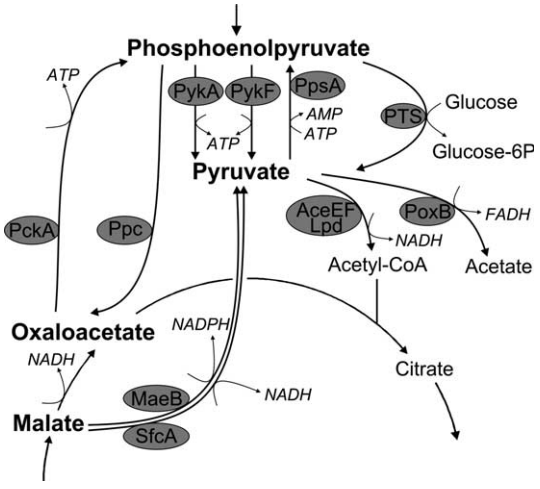

Fig. 3. The PEP–pyruvate–oxaloacetate node in aerobic *E. coli*. The reaction arrows point into the normal physiological direction. Abbreviations denote the gene products that catalyze a given reaction: PykA(F), pyruvate kinase(s); PpsA, PEP synthetase; PTS, phosphotransferase system (for glucose uptake); AceEF and Lpd, pyruvate dehydrogenase; PoxB, pyruvate oxidase; Ppc, PEP carboxylase; PckA, PEP carboxykinase; MaeB and SfcA, malic enzyme(s).

Mutants in *ppc* are strictly auxotrophic for TCA cycle intermediates such as succinate during growth on sugars [149] or glycerol [150], but appear to grow on non-repressing substrates such as acetate, lactate, malate, succinate, or ribose [149–151]. While the glyoxylate shunt could theoretically bypass the PEP carboxylase deletion during growth on glucose, this does not normally occur because (i) the shunt is subject to catabolite repression and (ii) the competing isocitrate dehydrogenase in the TCA cycle must be inactivated through phosphorylation, a process that is repressed by glucose [152] (compare also Section 3.3.). The glyoxylate shunt enzyme isocitrate lyase competes with the TCA cycle enzyme isocitrate dehydrogenase for the common substrate isocitrate. Beyond the aforementioned catabolite repression and enzyme phosphorylation, flux splitting at this key branch point between biosynthetic and energy-producing fluxes is also controlled at the level of isocitrate concentration [153]. Since the K_m of the lyase is rather high for isocitrate (0.6 mM), the flux is preferentially through the TCA cycle at low isocitrate concentrations (e.g. during growth on glucose), because the K_m of the dehydrogenase is two orders of magnitude lower [154]. Recently, two reports discussed *ppc* mutants with only mildly affected phenotypes on sole glucose [151,156]. At least in one case [156] the situation is somewhat unclear because the identical data were reported in another paper with the claim of succinate supplementation [157]. Generally, such *ppc* mutant phenotypes are probably due to physiological suppressor mutations in the glyoxylate shunt [158], and glyoxylate shunt flux was indeed shown in some suppressed *ppc* mutants (Perrenoud and Sauer, unpublished). The matter is further complicated by the apparent difference in transcriptional regulation of the glyoxylate shunt and the TCA cycle in different *E. coli* strains. Different from the typical K-12 strains, the glyoxylate shunt appears to be expressed in *E. coli* B during growth on glucose while the competing isocitrate dehydrogenase is repressed [159,160].

The anaplerotic function of PEP carboxylase can be fully restored by expression of (heterologous) pyruvate carboxylases in *ppc* mutants [161]. The metabolic consequence of such altered anaplerosis is an increased carbon flux to oxaloacetate, both aerobically and anaerobically during growth on glucose [146,161].

Anaerobically, this manipulation causes a shift in product formation from lactate to succinate. Under aerobic conditions, the surprising physiological consequence of this flux redirection is an about 50% increased biomass yield at an unaltered specific growth and glucose uptake rate [161]. An anaplerotic flux increase to oxaloacetate was also achieved by overexpressing the native PEP carboxylase with a rather similar outcome in glucose batch cultures: reduced acetate formation and a significantly increased biomass yield, albeit at reduced glucose uptake rate [162,163]. Collectively, these results strongly suggest that the anaplerotic reaction in *E. coli* is not optimized for unrestricted growth on glucose.

To ensure proper function under varying environmental, the tetrameric PEP carboxylase is subject to a complex allosteric regulation (Table 2), and considerable detailed knowledge on the molecular architecture and the functional domains of PEP carboxylase is available from the solved 3D structure [50] and amino acid replacement studies [164]. The enzyme is activated by acetyl-CoA, fructose-1,6-P (at low physiological concentrations), GTP, CDP, and long-chain fatty acids and inhibited by aspartate and malate [31,165–167]. The synergistic activation by the glycolytic intermediates acetyl-CoA and fructose-1,6-P [168] can be rationalized in terms of a feed-forward activation of oxaloacetate formation during sugar utilization. In this scheme, aspartate is a classical feedback inhibitor as the first biosynthetic product of oxaloacetate. On the basis of in vivo effector concentrations and by assessing the in vitro response of PEP carboxylase to the physiological concentration of each effector, it has been estimated that the in vivo enzyme activity was at most 15% of its maximal value [169].

3.2. *C4-decarboxylating reactions in* E. coli

Generally, two C4-decarboxylating routes exist for growth on dicarboxylic acids [4,143]. The first is that via the *pckA*-encoded PEP carboxykinase with a primar-

Table 2
Allosteric regulation of enzymes at the PEP–pyruvate–oxaloacetate node in *E. coli*

Enzyme	Known effector(s)[a]	Apparent $K_a$or K_i (mM)	Reference(s)
PEP carboxylase	Acetyl-CoA (+)	0.003–0.19[b]	[165,166,169]
	Fructose-1,6-P (+)	1.8	[169]
	GTP (+)	7.0	[169]
	Long-chain fatty acids (+)	0.02–1[c]	[167]
	Aspartate (−)	0.12–0.36[d]	[165,166,169]
	Malate (−)	0.4	[169]
	Citrate (−)	1–20[c]	[167]
	Succinate/fumarate	1–20[e]	[298]
PEP carboxykinase	ATP (−)	0.04	[177]
	PEP (−)	0.06	[177]
Malic enzyme (MaeB)	Oxaloacetate (−)	~0.01	[99]
	Acetyl-CoA (−)	0.07–0.35[f]	[99,299]
	cAMP (−)	0.4 (0.7)[g]	[300]
	NADH (−)	0.32–0.40	[99]
	NADPH (−)	0.14	[99]
Malic enzyme (SfcA)	Aspartate (+)	<0.5	[175]
	ATP (−)	0.6–1.5	[175]
	AD(M)P (−)	0.1–2.0[c]	[175]
	GT(D)P, ITP (−)		[175]
	cAMP (−)	0.3	[175]
	CoA (−)	0.35	[175]
PEP synthetase	Energy charge (+)	High values	[180,181]
	PEP (−)	<0.1	[181]
	Oxaloacetate (−)	0.3–0.6	[181]
	Oxoglutarate (−)	>0.6	[181]
	ADP (−)	0.2–2[c]	[181]
	AMP (−)	0.4–0.8	[181]

[a] (+) positive effector; (−) negative effector.
[b] The K_a of acetyl-CoA is 0.003 and 0.07 at 5.4 and 0 mM fructose-1,6-P, respectively [165].
[c] Effective range.
[d] The K_i of aspartate may increase up to 6 mM in the presence of 2.5 mM fructose-1,6-P [165].
[e] May have been converted to malate in the assay.
[f] Values obtained at around pH 7.0; at pH 9.3, 0.25 mM acetyl-CoA completely abolish activity [299].
[g] K_i at 2 mM (5 mM) malate, respectively.

ily gluconeogenic function during growth on dicarboxylic acids. The *E. coli* enzyme is ATP-dependent and occurs as a monomer [81]. As the second C4-decarboxylating reaction, malic enzymes convert malate to pyruvate under most physiological conditions [170], and the two isoenzymes encoded by *maeB* and *sfcA* preferentially use the redox cofactors NADP and NAD, respectively [171–173]. Both enzymes are used for growth on dicarboxylic acids but are not essential [172], because PEP may also be synthesized via malate dehydrogenase (malate: quinone oxidoreductase) and PEP carboxykinase. Deficiency of both routes is required to block growth on succinate or malate, e.g. in *pckA ppsA* double mutants [4,96]. Growth of a *pckA maeB sfcA* triple mutant, however, is only substantially impaired but not completely abolished [173]. Although both malic enzymes posses oxaloacetate decarboxylating activity [99,100] and a single point mutation suffices to confer such activity on PEP carboxykinase [174], *E. coli* does not seem to contain a specific oxaloacetate decarboxylase that might explain the slow growth of the triple mutant. Thus, residual growth of the triple mutant on dicarboxylic acids may be due to the somewhat leaky *pckA* mutation [112].

During growth on dicarboxylic acids, the NAD-dependent malic enzyme appears to have a primary, but non-essential function in gluconeogenesis, while the NADP-dependent isoenzyme is thought to supply the cell with NADPH from the decarboxylation of malate [4,173]. The K_m value of SfcA is much more favorable for malate decarboxylation (0.26 mM) than for pyruvate carboxylation (16 mM), but this NADH-dependent C3-carboxylating reaction can be used for anaerobic succinate production in mutants that are blocked in fermentative pyuvate metabolism [171].

Akin to the anaplerotic reaction, in vivo activity of the C4-decarboxylating reactions is modulated by allosteric effectors (Table 2), but also subject to strong transcriptional regulation. The tetrameric malic enzyme MaeB is inhibited by oxaloacetate, acetyl-CoA, NADH, and NADPH [99] and SfcA is activated by aspartate and inhibited by ATP (and its congeners) and CoA [175,176]. Despite its monomeric structure, PEP carboxykinase is allosterically inhibited by PEP and ATP (and other nucleotides) [177] but not by NADH [178], as was reported earlier [179]. Finally, PEP synthetase is inhibited strongly by PEP and to a lesser extent by AD(M)P, oxaloacetate, and oxoglutarate and stimulated by high energy charge values [180,181], which would, if expressed, diminish its activity during sugar catabolism. Together with the inhibition of the pyruvate dehydrogenase complex at high energy charge, this regulation scheme ensures appropriate partitioning between anaplerotic/gluconeogenic (PEP synthetase) and catabolic (pyruvate dehydrogenase complex) flux during growth on lactate, pyruvate, or alanine when both enzymes compete for pyruvate. Notably, the PEP inhibition of PEP synthetase overrides the influence of the energy charge. Independent of the energy charge, 0.1 mM PEP suffice to affect predominant formation of acetyl-CoA (catabolism) in in vitro mixtures of PEP synthetase and pyruvate dehydrogenase complex [180].

At the transcriptional level, the gluconeogenic *pckA*, *ppsA*, *maeB*, and *sfcA* have long been known to be subject to glucose repression [5,142,182], thus reducing the risk of potential futile cycling with the glycolytic enzymes. Some of the genes at the node are known targets of global transcriptional regulators, e.g. *pckA* is repressed in a Crp-dependent manner and repression is relieved at low cAMP levels [94]; *ppsA* and *pckA* are induced and *pykF* is repressed by the catabolite repressor/activator Cra (formerly known as FruR) [183]. Additionally, *pckA* is also strongly induced at the onset of stationary phase [5,96], possibly through accumulated by-products such as acetate. More recently, accumulating global transcript data reconfirmed the glucose repression of gluconeogenic genes (i.e. *pckA*, *ppsA*, *maeB*, *sfcA*, and *fbp*) in minimal and, at least for *pckA*, in complex media [93,94,184]. Expression levels were somewhat higher in complex media when no glucose was supplemented [185]. As may be expected from the external supply of TCA-based amino acids, *ppc* expression is lower in glucose complex medium than in glucose minimal medium [186]. Under anaerobic condition in complex medium, expression of *sfcA* and *ppsA* was significantly higher in xylose-containing compared to glucose-containing media [187], but the logic is presently not understood.

Beyond descriptive transcriptome analyses, recent efforts attempt to elucidate metabolism-wide regulation processes that underlie the switch from glucose to acetate as the sole carbon source [188]. In particular, *pckA* expression was activated immediately after the switch within the first 5 min. Using network component analysis, the effects of multiple regulatory pathways could be deconvoluted from the data, indicating that increased *ppsA* and *pckA* expression was mediated by the global transcription factors Cra (formely FruR) and the CRP-cAMP complex, respectively [188]. The latter scheme is supported by the absence of glucose-based *pckA* repression in a CRP mutant [94], and both are fully consistent with the qualitatively known mechanisms of *ppsA* and *pckA* induction by both factors, which is absent in the presence of glucose [182,183].

3.3. Carbon fluxes and regulation at the PEP–pyruvate–oxaloacetate node in E. coli

Despite the accumulated molecular and physiological knowledge, a more comprehensive and quantitative understanding of the structurally entangled set of reactions in the PEP–pyruvate–oxaloacetate node is

required, e.g. on the in vivo relevance of the various genetic and allosteric regulation mechanisms. This lack of understanding may be illustrated by the apparently insufficient expression level of several genes of the PEP–pyruvate–oxaloacetate node for optimal unrestricted growth on glucose [162,163] or on gluconeogenic substrates such as pyruvate and succinate [145]. To foster understanding, obviously different types of data must be integrated, and first efforts along these lines include comparison of metabolic flux and mRNA expression data under glucose versus alternative carbon source conditions [93,187]. The rather high correlation between both quantities suggests that flux is primarily controlled at the transcriptional level, but this conclusion should not be generalized [189,190] (compare also Section 5). Knowledge about the in vivo operation of pathways and reactions is of key relevance in this context because it represents the culmination of all genetic and allosteric regulation. Since in vivo reaction rates are per se non-measurable quantities, they must be inferred from physiological and ^{13}C-labelling data by methods of metabolic flux analysis [191,192]. By considering metabolism as a system rather than individual reactions, flux analysis can provide new insights into the function of particular reactions. One such example at the PEP–pyruvate–oxaloacetate node is the local bypass of catabolic flux around pyruvate kinase deletion via PEP carboxylase and malic enyme in *E. coli* [144] (Fig. 4) but not in *B. subtilis* [193]. Jointly, both enzymes contribute to catabolism, which goes well beyond their individual functions in anaplerosis and gluconeogenesis.

Another prominent example for new insights into the function of particular pathways by metabolic flux analysis are ATP-dissipating futile cycles – resulting from simultaneous activity of glycolytic and gluconeogenic reactions – that, based on biochemical "common sense" as well as qualitative genetic and allosteric regulation data, would be expected to be absent or operate only at low level. In particular the PEP carboxylase/PEP carboxykinase pair of *E. coli*, however, was found to catalyze substantial "futile" fluxes under strict glucose limitation in slow-growing chemostat cultures [144,194,195]. Such "futile" ATP dissipation may be quite substantial, amounting to up to 8% of the total cellular energy budget [190], which is surprising because *pckA* expression is not higher in slow than in more rapidly growing chemostat cultures [196]. A detailed kinetic analysis strongly suggests that this in vivo PEP carboxykinase activity is mostly modulated at the level of enzyme activity by the changes in PEP and oxaloacetate concentrations, rather than by changes in the concentration of the other allosteric regulator ATP [190].

How relevant is such futile cycling for the overall cellular operation? Installing an artificial futile cycle by jointly overexpressing pyruvate carboxylase and PEP carboxykinase, Chao and Liao [197] demonstrated that

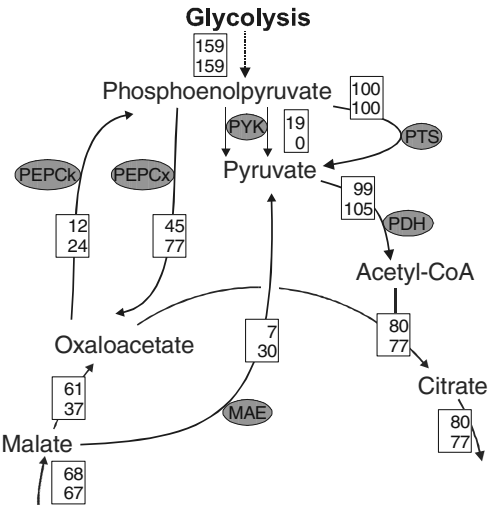

Fig. 4. Distribution of metabolic fluxes at the glycolysis-Krebs cycle interface in wild-type *E. coli* (top entry in the boxes) and a knockout mutant of both pyruvate kinase isoenzymes (bottom entry). Flux values are relative to the specific glucose uptake rate and were obtained from glucose-limited continuous cultures at a growth rate of 0.4 h^{-1} [144]. Note the local bypass of pyruvate kinase via malic enzyme and PEP carboxylase. *Abbreviations:* see legend to Fig. 2.

the specific rate of growth was insensitive to increasing ATP-dissipation and that the cells counteracted the effect by increasing the rates of oxygen and substrate consumption as well as by-product secretion; at the expense of the biomass yield. More generally, this suggests that the associated ATP dissipation is nor overly critical for vital cell functions.

Lastly, it should be realized that the traditional metabolic pathways are biochemical models that do not necessarily represent the entire intracellular reality. Beyond its recognized anaplerotic function during growth on acetate, accumulating evidence from ^{13}C-labelling experiments suggests that the glyoxylate shunt counteracts the metabolic impact of several mutations (i.e. *ppc*, *pgi*, *pckA*) [156,190,195]. This in vivo activity was mostly seen under strict glucose limitation in slow-growing chemostat cultures when catabolite repression of the shunt is expected to be less stringent or absent [198]. At least in the case of *pgi* (phosphoglucose isomerase) mutants, however, the glyoxylate shunt is also active under glucose excess batch conditions [11,199]. While the shunt may be interpreted as a substitute anaplerotic reaction in *ppc* mutants, its activity in *pgi* or *pckA* mutants is less clear. Additionally, these results open the question on whether the glyoxylate shunt is indeed inactive in glucose-grown wild-type cultures. This question is not routinely addressed in metabolic flux analyses because absence of the shunt is a priori assumed in most network models (but not the above ones) that are used to interpret the ^{13}C-labelling pattern [144,156,190,195,200] – hence can often not be identified to be active.

Using a more comprehensive network model, in vivo glyoxlate shunt activity was indeed shown in slow-growing wild-type *E. coli* in glucose-limited chemostat cultures akin to those mentioned above, while it was inactive in rapidly growing batch or chemostat cultures [11]. The metabolic system-wide perspective of flux traffic then revealed that glyoxylate shunt activity cannot be understood individually, but rather that it functions in combination with PEP carboxykinase in the so-called PEP–glyoxylate cycle (Fig. 5). This novel cycle catalyzes complete oxidation of carbohydrates to CO_2, a property that was previously considered to be exclusive for the TCA cycle [12]. Conjoint operation of PEP carboxykinase and the glyoxylate shunt in this bi-functional catabolic and anabolic cycle is in sharp contrast to their 'textbook' function of gluconeogenesis and anaplerosis, respectively. The PEP–glyoxylate cycle might then also explain the unexpected activity of the glyoxylate shunt and the significant phenotype in a *pckA* mutant during glucose-limited chemostat cultivation [190], since the *pckA* mutation effectively disrupts the cycle. The metabolic function of the PEP–glyoxylate cycle is not directly obvious because it is functionally redundant with the PEP carboxylase and the TCA cycle. One physiological function may be decoupling of catabolism from NADPH formation that would otherwise occur in the TCA cycle. This hypothesis is fully consistent with the activity of the PEP–glyoxylate cycle in *pgi* mutants that produce a large NADPH excess by extensive use of the pentose phosphate pathway [11,199].

3.4. Metabolic engineering of the node

As a consequence of its central metabolic position, the PEP–pyruvate–oxaloacetate node has been the focus point of several metabolic engineering attempts to improve biotechnological production processes. One such case is fermentative succinate production with *E. coli*, which requires extensive anaplerotic fluxes. Several metabolic engineering routes have been implemented successfully, including overexpression of the native PEP carboxylase [201], the NAD-dependent malic enzyme ScfA (in a *pfl ldhA* mutant) [202], and a heterologous PEP carboxykinase (in a *ppc* mutant) [203]. Different strategies have been applied to obtain metabolically engineered strains of *E. coli* for efficient conversion of glucose to pyruvate. Strains with mutations in genes encoding the subunits E1 or E2 (*aceE* and *aceF*, respectively) of the pyruvate dehydrogenase complex and with a deletion of the PEP carboxylase gene *ppc* exhibited significant pyruvate accumulation [204]. The most promising production strains (catalyzing a conversion of 1 mol glucose to 1.78 mol pyruvate; final pyruvate titers of >900 mM) are completely blocked in their ability to convert pyruvate into acetyl-CoA, PEP, acetate and lactate. This was realized by chromosomal deletion of the genes coding for the pyruvate dehydrogenase complex (*aceEF*), pyruvate formate lyase (*pflB*), PEP synthetase (*pps*), pyruvate: quinone oxidoreductase (*poxB*), and lactate dehydrogenase (*ldhA*) (M. Bott, personal communication; [205,206]). Another approach for the efficient conversion of glucose to pyruvate was to combine mutations to minimize ATP yield, cell growth, and CO_2 production with mutations that eliminate acetate production and fermentation products (*E. coli* W3110 Δ*focA*–*pflB*Δ*frdBC*Δ*ldhA*Δ*atpFH* Δ*adhE*Δ*sucA pox*::FRT Δ*ackA*) [207].

A particular focus area for metabolic engineering of the node was production of aromatic compounds, which starts with the synthesis of 3-deoxy-D-arabino-heptulosonate-7P (DHAP) from the fusion of the erythrose-4P and PEP precursors [147,148,208–210]. Since PEP is also required for PTS-based glucose uptake and for anaplerosis via PEP carboxylase (Fig. 3), at least one of the precursors is not readily available for high-level production of aromatic compounds in *E. coli*, and various strategies for enhancing PEP availability were described. Beyond attempts to reduce the drain of PEP to pyruvate through deletion of pyruvate kinase(s) and installing a non-PTS for glucose uptake, several efforts focussed on relieving the

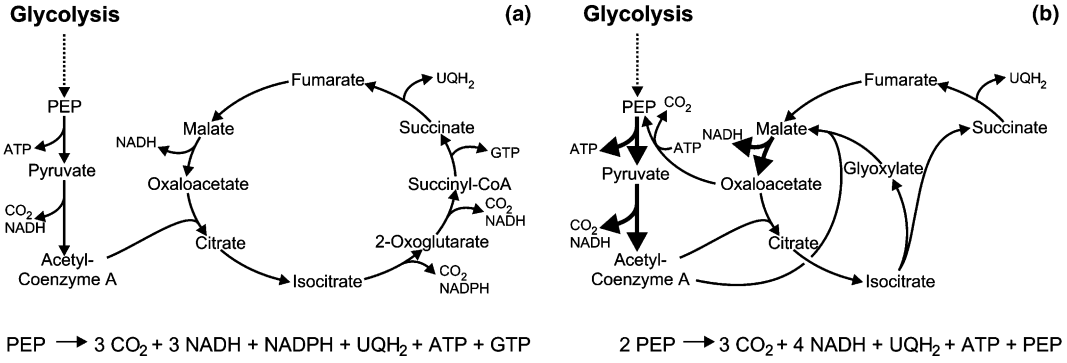

Fig. 5. Stoichiometry of the TCA cycle (a) and the PEP–glyoxylate cycle (b) in *E. coli* [11]. Solid arrows indicate reactions that are required twice per cycle.

stoichiometric problem by increasing gluconeogenic fluxes to PEP [148,209]. Simple deletion of *ppc* in an overproducing mutant stimulates aromatics production but also secretion of undesired by-products such as acetate, hence is not an appropriate strategy [211]. Apparently, the glyoxylate shunt was either not active in these mutants (compare also Section 3.1.) or it is insufficient to counteract the strong overflow metabolism to acetate. Overexpression of the gluceoneogenic PEP synthetase, in contrast, has a pronounced effect on the achievable aromatics yield on glucose. In combination with overexpression of the erythrose-4P supplying transketolase, PEP synthetase overexpression allowed to achieve near theoretical yields in a recombinant strain with a deregulated biosynthetic pathways to DHAP [212,213].

4. The PEP–pyruvate–oxaloacetate node in *B. subtilis*

4.1. Anaplerosis and C4-decarboxylation reactions in B. subtilis

The aerobic, spore-forming *B. subtilis* has become the Gram-positive model bacterium, with extensive international (post)genomic projects [214,215] and of significant biotechnological relevance [216]. Nevertheless, comparatively little biochemical data are available on the C3-carboxylating and C4-decarboxylating enzymes and most of our physiological knowledge dates back to the seminal paper by Diesterhaft and Freese [67]. The major difference to *E. coli* is replacement of PEP carboxylase with pyruvate carboxylase as the C3-carboxylating anaplerotic reaction (Fig. 6), which is a typical bacterial variant of this enzyme with a deduced molecular mass of 127.7 kDa. Without a functional glyoxylate shunt, pyruvate carboxylase is the sole anaplerotic reaction, thus *B. subtilis* cannot grow on substrates that are metabolized via acetyl-CoA [217]. The 3.4 kb monocistronic *pycA* gene is constitutively expressed, but pyruvate carboxylase is subject to strong allosteric activation by acetyl-CoA [67,105]. The lack of this allosteric activation appears to cause the low in vivo activity during co-metabolism of glucose and citrate [218]. In mutants with a defective TCA cycle, pyruvate carboxylase is apparently incapable to sustain sufficiently high intracellular oxaloacetate pools for amino acid biosynthesis under many conditions, hence such mutants must be supplemented with aspartate solely for this reason [219].

Pyruvate carboxylase mutants of *B. subtilis* are incapable of growth on substrates that enter upstream of pyruvate, unless supplemented with TCA cycle replenishing co-substrates [67]. The reverse PEP carboxykinase reaction could, in principle, bypass the lesion by catalyzing C3-carboxylation of PEP, as has been

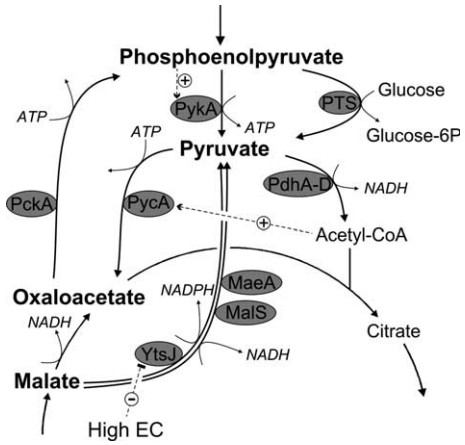

Fig. 6. The PEP–pyruvate–oxaloacetate node in *B. subtilis*. The reaction arrows point into the normal physiological direction. Allosteric regulation is indicated by thin, hatched arrows. *Abbreviations* (gene products that catalyze a given reaction): PykA, pyruvate kinase; PTS, phosphotransferase systems (for glucose uptake); PdhABCD, pyruvate dehydrogenase complex; PycA, pyruvate carboxylase; PckA, PEP carboxykinase; MaeA, MalS, and YtsJ, malic enzyme(s); EC, energy charge.

suggested for some bacterial species [83,88,92], but the physiological equilibrium position strongly favors C4-decarboxylation with a $\Delta G^{0'}$ of -12.4 kJ M^{-1} [220,221]. The normal C4-decarboxylating function of PEP carboxykinase in the gluconeogenic supply of PEP during growth on TCA cycle intermediates has been proven by the lethal phenotype of a *pckA* mutant on malate [67]. More recently, ^{13}C-experiments demonstrated, however, that PEP is indeed carboxylated to oxaloacetate via PEP carboxy- kinase in certain deletion mutants [222]. In the background of a pyruvate kinase mutant, the normally gluconeogenic PEP carboxykinase contributes significantly to glucose catabolism in carbon-limited chemostat cultures. This flux was from PEP to oxaloacetate and not via a potential pyruvate kinase-like side activity that is displayed by some PEP carboxykinases [90]. The reversed flux against the normal thermodynamic equilibrium was probably facilitated by the about 50-fold increased PEP concentrations in pyruvate kinase mutants [223]. In contrast to wild-type 168, some pyruvate carboxylase-deficient *B. subtilis* strains with the mutation can slowly grow on glucose [222]. Such strain differences may be related to the actual PEP levels, but the general conclusion is that the kinetics of simultaneous anaplerotic PEP carboxylation and ATP generation by PEP carboxykinase are not favorable for aerobic growth. Thus, the picture of a bi-functional *B. subtilis* PEP carboxykinase emerges with a primary C4-decarboxylating function in the classical gluconeogenic direction and a minor catabolic C3-decarboxylating role, at least in certain mutants.

Apart from the recently acquired molecular knowledge about transcriptional control of the monocistronic *pckA* gene by the carbon catabolite regulators CcpA [224,225] and CcpN [226], very little data are available on the *B. subtilis* PEP carboxykinase. Different from the monomeric *E. coli* enzyme, the deduced molecular mass of 43.5 kDa and the apparent native molecular mass above 100 kDa [67] suggest a di- or tetrameric structure of PEP carboxykinase in *B. subtilis*. At least fructose-1,6P, glucose-6P or AMP exert no allosteric effects on the purified enzyme [67], but other typical effectors such as PEP and ATP (compare 3.2.) have not yet been investigated.

Bacillus subtilis contains four paralogues encoding putative malic enzymes. Based on genomic data, *mleA* was strongly suggested to encoded a malolactic rather than a malic enzyme [227]. The remaining three enzymes fall into two groups, the 43.5 kDa YtsJ and the larger, 62–64 kDa MalS and MaeA (formerly *ywkA*) (Fig. 6). The former two are transcribed from monocistronic genes, while *maeA* is organized in an operon with the functionally uncharacterized *ywkB*. YtsJ is apparently the major malic enzyme because only *ytsJ* mutants grow significantly slower than the wild-type on malate or other TCA cycle intermediates, while *maeA* and *malS* single or double mutants are indistinguishable from their parent [105]. The 1.2 kb *ytsJ* gene is constitutively expressed during exponential growth on either glucose or malate [105] and encodes the NADP-dependent malic enzyme (Doan, Lerondel, and Aymerich, unpublished data). Although specifically induced by malate via the MalK/MalR (formerly YufL/YufM) two-component system and biochemically characterized as a primarily NAD-dependent malic enzyme, *maeA* mutants grow normally on malate [105]. Hence, MaeA appears to participate in a more specialized biological process that is primarily relevant in the presence of malate. The constitutive expression pattern of *malS* under gluconeogenic and glycolytic conditions provides no hint at its function [105], thus the role of MalS remains obscure. The original biochemical assignment of malic enzyme co-factor specificity was probably based on MaeA because it was purified from *B. subtilis* grown on malate [67]. Using crude cell extracts, Ohné [228] established that the specific activity of malic enzyme is not affected by any of 10 different intracellular metabolites tested but is only inhibited by high energy charge values through ATP. These enzyme data relate probably to the YtsJ isoform because the cultures were grown in rich medium. At least in the closely related *B. stearothermophilus*, purified NAD(P)-dependent malic enzyme catalyzes also decarboxylation of oxaloacetate [118].

4.2. Carbon fluxes and regulation at the PEP–pyruvate–oxaloacetate node in B. subtilis

Given constitutive *ytsJ* expression, in vivo malic enzyme fluxes may not be overly surprising in glucose-grown *B. subtilis* [221,222] and other bacilli [229] (Table 3). In combination with pyruvate kinase, malic enzyme(s) constitutes the so-called pyruvate shunt that may have substantial in vivo activity in the range of 10–30% of the glucose uptake rate, e.g. during carbon-limited grow on glucose/acetoin, glucose/acetate, and glucose/gluconate [218] or during rapid, phosphate-limited growth [9]. Substantial PEP carboxykinase fluxes on glucose are more surprising because *pckA* transcription is very weak in the presence of glucose [226], but extracellular glucose levels are extremely low in carbon-limited chemostat cultures. Simultaneous operation of PEP carboxykinase, pyruvate kinase and pyruvate carboxylase thus constitutes an ATP-dissipating futile cycle (Fig. 6). Although not overly relevant in terms of the total cellular energy balance, the molecular flux could be up to 25% of the glucose uptake rate in slow-growing glucose-

Table 3
Metabolic fluxes in the PEP-pyruvate oxaloacetate node of *B. subtilis* under different environmental conditions

Cultivation condition	PEP carboxykinase (%)	Malic enzymes (%)	Pyruvate carboxylase (%)	Malate dehydrogenase (%)	Reference
Batch culture, glucose[a]	8	6	32	37	[230]
Chemostat cultures[b]					
Glucose-limited, $D = 0.1$	25 (23)[c]	0 (16)	43 (54)	91 (64)	[9]
Glucose-limited, $D = 0.4$	6 (4)[c]	3 (9)	32 (35)	64 (35)	[9]
Ammonia (or phosphate)-limited, $D = 0.1$	21 (0)	11 (0)	48 (11)	100 (7)	[9]
Ammonia (or phosphate)-limited, $D = 0.1$	13 (1)	3 (27)	36 (40)	54 (−11)	[9]
Glucose/acetate (or acetoin)-limited, $D = 0.1$	19 (20)	15 (9)	53 (47)	173 (125)	[218]
Glucose/citrate-limited, $D = 0.10$	21	0	8	154	[218]

Values are percentages of the specific glucose uptake rate.
[a] Wild-type 168.
[b] Industrial riboflavin-producing strain (influence of riboflavin production on these fluxes is probably minor).
[c] Values in parenthesis are from a wild-type-like riboflavin producer [221].

limited cultures [9,221] and up to 8% in glucose-excess batch cultures of *B. subtilis* [230] (Table 3).

Switching from glycolytic to gluconeogenic growth, malic enzyme(s) and PEP carboxykinase become the key enzymes in reverting the carbon flow through the PEP–pyruvate–oxaloacetate node. Transcriptional regulation appears to play only a partial role in controlling this major flux redirection because just *pckA* is repressed by glycolytic carbon sources [224–226]. The major malic enzyme YtsJ [105] and the glycolytic *pykA* and *pycA*, in contrast, are expressed constitutively [67,105,225,231]. *pdhABCD* is slightly induced by glucose in succinate-glutamate media [225], but also higher expressed in malate than in glucose minimal media [105]. Thus, allosteric regulation appears to play a major role in the glycolytic-gluconeogenic switch of *B. subtilis*. In particular the strong allosteric activation of pyruvate carboxylase by acetyl-CoA [67] and the inhibition of malic enzymes by high energy charge [228] are expected to be major control factors in this flux redirection. Unfortunately, essentially nothing is known about the energy charge during growth on malate. In contrast to all other investigated bacilli that contain allosteric pyruvate kinases (activated by AMP and ribose-5-P and inhibited by ATP and fructose-1,6-diP) [232], the activity of the *B. subtilis* enzyme is not modified by AMP, ATP, or fructose-1,6-P but only activated by its substrate PEP [231].

5. The PEP–pyruvate–oxaloacetate node in *C. glutamicum*

Corynebacterium glutamicum is an aerobic, Gram-positive organism that grows on a variety of sugars and organic acids and is widely used in the industrial production of amino acids, particularly L-glutamate and L-lysine [233]. Due to the importance for the distribution of the carbon flux within the metabolism and for the precursor supply for amino acid synthesis, the PEP–pyruvate–oxaloacetate node of this organism (Fig. 7) has been intensively studied and much attention has been focused on some of the enzymes involved [77,80,84,234–236].

The oxidative decarboxylation of pyruvate for fueling the TCA cycle with acetyl-CoA in *C. glutamicum* has been generally attributed to the pyruvate dehydrogenase complex. Activity of this complex has been detected in various strains of *C. glutamicum* [237–240], however, only little effort has been devoted to the study of the complex at the molecular and structural levels. According to activity determinations in cell-free extracts, the *C. glutamicum* pyruvate dehydrogenase complex is not subject to any significant regulation that could modulate its activity [237,238]. This is surprising since in other bacteria and in eukaryotic organisms the activity of the complex is controlled by various metabolites [217,241–244]. However, there is a need for purification and biochemical

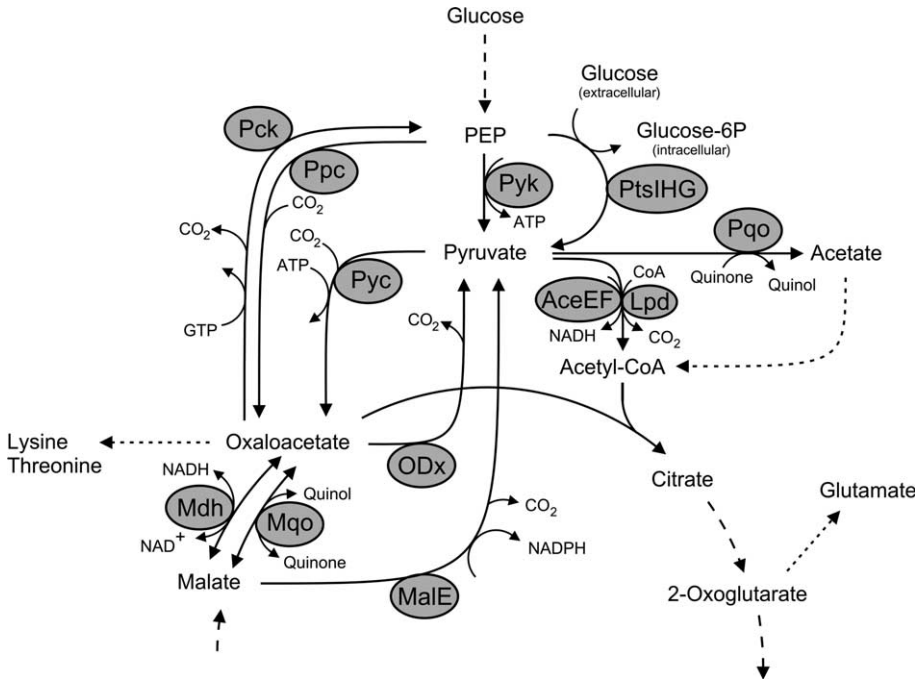

Fig. 7. The PEP–pyruvate–oxaloacetate node in *C. glutamicum*. Abbreviations denote the gene products that catalyze a given reaction: AceEF, subunits E1 and E2 of the pyruvate dehydrogenase complex; Lpd, subunit E3 of the pyruvate dehydrogenase complex; MalE, malic enzyme; Mdh, malate dehydrogenase; Mqo, malate: quinone oxidoreductase; ODx, oxaloacetate decarboxylase (gene not annotated); Pck, PEP carboxykinase; PtsIHG, phosphotransferase system; Pqo, pyruvate: quinone oxidoreductase; Pyc, pyruvate carboxylase; Pyk, pyruvate kinase.

analysis of the *C. glutamicum* pyruvate dehydrogenase complex and subsequently, the question for the control of its reaction and thus of the carbon flux into the TCA cycle can be clarified.

The reaction of the pyruvate dehydrogenase complex may be bypassed by the combined activities of pyruvate: quinone oxidoreductase, acetate kinase and phosphotransacetylase. The presence of the pyruvate: quinone oxidoreductase in *C. glutamicum* has been deduced from the annotation of its genome (Accession Nos. NC_003450 and BX927147; [245,246]) and recently, the respective activity has been detected and the enzyme has been purified and biochemically characterized (M. Schreiner and B.J. Eikmanns, manuscript submitted). The enzyme catalyzes the oxidative decarboxylation of pyruvate with a naphtoquinone as electron acceptor and is activated by detergents and phosphatidylglycerol. Acetate kinase and phosphotransacetylase are constitutively expressed in *C. glutamicum* and catalyze the formation of acetyl-CoA from acetate [247]. However, since the acetate kinase reaction requires ATP, the bypass of the pyruvate dehydrogenase complex reaction via pyruvate:quinone oxidoreductase, acetate kinase and phosphotransacetylase would be energetically unfavorable. Moreover, compared to the pyruvate dehydrogenase complex, the pyruvate: quinone oxidoreductase has a very low affinity for pyruvate (K_m values of 0.8 and 30 mM, respectively) ([240]; M. Schreiner and B.J. Eikmanns, manuscript submitted) and regarding the intracellular concentration of 0.5–0.8 mM pyruvate [248], it seems questionable whether pyruvate: quinone oxidoreductase significantly contributes to the oxidative pyruvate decarboxylation under the conditions *C. glutamicum* generally is cultivated. Thus, the true physiological function of this enzyme still remains to be elucidated.

In contrast to many other organisms, *C. glutamicum* possesses both a PEP carboxylase and a pyruvate carboxylase as anaplerotic enzymes [29,30,77,234] and both are present during growth and amino acid production on glucose [80,249–251]. Aside from the two C3-carboxylating enzymes, *C. glutamicum* possesses three C4-decarboxylating enzymes converting oxaloacetate or malate to PEP or pyruvate, i.e. PEP carboxykinase, malic enzyme and oxaloacetate decarboxylase (Fig. 7 and Table 1) [117,138,235,252]. Carboxylating activity of these three enzymes and thus a participation in anaplerosis during growth of *C. glutamicum* on glucose has been excluded [80]. Together with the anaplerotic PEP carboxylase and pyruvate carboxylase, five enzymes have been identified as directly interconverting C3 with C4 units at the PEP–pyruvate–oxaloacetate node of this organism. This surprising diversity of carboxylating and decarboxylating enzymes at the corynebacterial PEP–pyruvate–oxaloacetate node raises the question for the significance of all these reactions for growth and the biotechnologically relevant amino acid production.

In the following section, the present state of knowledge on activity and regulation of the C3-carboxylating (anaplerotic) and the C4-decarboxylating enzymes at the PEP–pyruvate–oxaloacetate node of *C. glutamicum* and on expression and regulation of the respective genes are discussed. The relevant regulatory characteristics of the enzymes are summarized in Table 4. Furthermore, we discuss what is known about the parallel and bidirectional fluxes, flux partitioning and the global control of the node.

5.1. *Anaplerosis in* C. glutamicum

PEP carboxylase has been known to be present with high specific activities in all *C. glutamicum* strains tested [29,30,234,236,253–255] and for a long time, the anaplerotic function in glucose-grown *C. glutamicum* cells generally has been attributed only to this enzyme [233,256,257]. It was shown to be activated and inhibited by several effectors (Table 4). The relatively high activity, the regulatory properties and also carbon flux studies suggested a key role of PEP carboxylase in the carbon flow to amino acids derived from the TCA cycle and therefore, the enzyme was proposed to be an important target in breeding *C. glutamicum* amino acid-producing strains [234,257].

The PEP carboxylase gene (*ppc*) from *C. glutamicum* was cloned and sequenced [258,259]. It is organized in a glycolytic gene cluster together with the genes encoding glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase and triosephosphate isomerase [259], and is transcribed in an operon together with the latter two genes [260] The construction and comparative analysis of defined PEP carboxylase-negative mutants of *C. glutamicum* revealed that the enzyme is dispensable for growth and lysine production [252,255]. These results indicated that (an) additional anaplerotic enzyme(s) operate(s) in *C. glutamicum* and genetic experiments as well as in vivo ^{13}C-labelling experiments with subsequent ^{1}H NMR analyses identified the alternative anaplerotic reaction in *C. glutamicum* to be a PEP or pyruvate carboxylation [261,262]. Further investigations resulted in the detection of pyruvate carboxylase activity and in the cloning and characterization of the respective gene (*pyc*) and its expression [77,80]. Later, the pyruvate carboxylase reaction was identified as the major bottleneck for glutamate and lysine production by *C. glutamicum* [249] and thus, the pyruvate carboxylase enzyme rather than PEP carboxylase became one of the primary targets for the optimization of fermentative amino acid production [263].

The pyruvate carboxylase enzyme of *C. glutamicum* was very unstable and therefore it could be detected reliably only in an in situ enzyme assay using permeabilized

Table 4
Known effectors of enzymes of the PEP–pyruvate–oxaloacetate node in *C. glutamicum*

Enzyme	Known effector(s)[a]	Apparent $K_a$or K_i (mM)	Reference(s)
PEP carboxylase	Citrate (−)	10	[30,155,234,253]
	Succinate (−)	3.6	
	Malate (−)	6.7	
	Aspartate (−)	0.04–0.18	
	Acetyl-CoA (+)	0.04–0.2	
	Fructose-1,6-P (+)	~0.2	
	NH_4-glutamate (−)	40	
Pyruvate carboxylase	ADP (−)	2.6	[77,250]
	AMP (−)	0.75	
	Acetyl-CoA (−)	0.11	
	Aspartate (−)	~15	
PEP carboxykinase	ATP (−)	~0.1	[235]
Malic enzyme	Oxaloacetate (−)	>10	[117]
	Glutamate (−)	>200	
	NH_4^+(+)	1.25	
Oxaloacetate decarboxylase	ADP (−)	1.2	[138]
	GDP (−)	1.8	
	CoA (−)	2.4	
	Succinate (−)	2.8	
Pyruvate kinase	AMP (+)	<2 mM	[236,253,255]
	ATP (−)	~0.4	
	GTP (−)	1	

[a] (+) positive effector; (−) negative effector.

cells and so far, it was not possible to prepare a pure and active pyruvate carboxylase from *C. glutamicum*. Characterization of pyruvate carboxylase activity in permeabilized cells revealed that the enzyme is effectively inhibited by several metabolites (Table 4). Further investigations showed that pyruvate carboxylase represents a biotin-containing enzyme of about 125 kDa and that its synthesis in *C. glutamicum* is about threefold upregulated by lactate as the carbon source in the growth medium [80,250].

The *C. glutamicum* pyruvate carboxylase gene (*pyc*) has been isolated and characterized [80,264]. The deduced polypeptide consists of 1140 amino acids (123.1 kDa) with typical binding sites for ATP and pyruvate and a biotin-carrier domain, and it shows up to 63% identity to known pyruvate carboxylase enzymes from other organisms. Analysis of defined pyruvate carboxylase- and PEP carboxylase-negative single and double mutants of *C. glutamicum* showed that pyruvate carboxylase is essential for growth on lactate and pyruvate and that no further anaplerotic enzymes for growth on carbohydrates exist apart from pyruvate carboxylase and PEP carboxylase [80]. Furthermore, the results indicated that the two enzymes could at least partially replace each other as anaplerotic enzymes for growth of *C. glutamicum* on glucose. For maximal growth rate and yield, however, the presence of pyruvate carboxylase rather than PEP carboxylase was essential [80]. Labelling studies with ^{13}C substrates and subsequent NMR analyses revealed that activities of both enzymes are simultaneously present in glucose-growing cells of *C. glutamicum* with the pyruvate carboxylase enzyme contributing about 90% of the total oxaloacetate synthesis ([10]; see also Section 5.3.). This latter result underlines the importance of pyruvate carboxylase for growth of and amino acid production by *C. glutamicum*. However, the presence of both enzymes might increase the flexibility of the organism when faced with different carbon sources or substrate mixtures. The activities of pyruvate carboxylase and PEP carboxylase are controlled by different effectors (see Table 4) and the two enzymes may have different affinities to HCO_3^- and thus, the use of the one or the other reaction might be correlated to specific conditions.

5.2. C4-decarboxylation reactions in C. glutamicum

As in most other organisms able to grow on TCA cycle intermediates, the initial reaction of gluconeogenesis in *C. glutamicum* is accomplished by the C4-decarboxylating PEP carboxykinase (Fig. 7) [84,235,252]. While microbial enzymes often use ATP as phosphate donor (see Table 1), the *C. glutamicum* enzyme has been shown to be highly specific for GTP [235,252] and thus represents a notable exception. The *C. glutamicum* PEP carboxykinase has been purified and kinetic analysis revealed that oxaloacetate-forming activity of the enzyme is inhibited by ATP (Table 4). This finding already suggested that the enzyme

under physiological conditions mainly functions in gluconeogenesis and not in anaplerosis.

The PEP carboxykinase gene *pck* from *C. glutamicum* has been characterized and used for construction of recombinant strains with no or altered PEP carboxykinase activities [84]. The deduced PEP carboxykinase consists of 610 amino acids (66.9 kDa) and shows almost no similarity to ATP-dependent but up to 64% identity with GTP-dependent PEP carboxykinases from eukaryotic organisms. The expression of the *pck* gene is regulated by the carbon source in the growth medium, resulting in about threefold higher specific activities in acetate- or lactate-grown cells than in glucose-grown cells [84]. Independently, DNA microarray and quantitative RT-PCR experiments substantiated the acetate-dependent transcriptional regulation of the *C. glutamicum pck* gene [265]. A growth phase-dependent regulation of *pck*, as it has been shown for other bacterial *pck* genes (e.g. [5,6]), has not been observed in *C. glutamicum* [84].

The gluconeogenic function of PEP carboxykinase in *C. glutamicum* has been proven by the analysis of a defined PEP carboxykinase-negative mutant [84]. Such a mutant was able to grow on glucose but not on substrates requiring gluconeogenesis. The inability of the mutant to grow on acetate or lactate furthermore indicated that PEP carboxykinase is the only enzyme responsible for PEP synthesis from TCA cycle intermediates and that it cannot be functionally replaced by the combined activities of malic enzyme or oxaloacetate decarboxylase with PEP synthetase. The presence of a PEP synthetase in *C. glutamicum* was proposed for some strains [236,257], however, the result mentioned above and the fact that a pyruvate carboxylase-negative mutant was unable to grow on lactate [80], argues against the presence of a functional PEP synthetase. This enzyme in combination with PEP carboxylase should have bypassed the pyruvate carboxylase reaction in the respective mutant and should have allowed growth on lactate and pyruvate.

Malic enzyme activity has been detected in *C. glutamicum* under various growth conditions [237,238,257]. In contrast to the situation in *E. coli* or *B. subtilis*, there is only one malic enzyme present in *C. glutamicum* [117]. The enzyme has been purified from *C. glutamicum* and biochemically analyzed [117]. It is strictly specific for NADP, is activated by NH_4^+ and slightly inhibited by oxaloacetate and glutamate. The maximal velocity of the decarboxylation reaction was about fivefold higher than the carboxylating activity. This result and a relatively low affinity for pyruvate (K_m = 13.4 mM) suggest that the in vivo function is the decarboxylation of malate linked to NADPH generation rather than the reverse carboxylation of pyruvate [117].

The malic enzyme gene (*malE*) has been characterized and used for the construction of malic enzyme-negative and *malE*-overexpressing strains of *C. glutamicum* [117]. The deduced product of *malE* consists of 392 amino acids (40.9 kDa) and is up to 69% identical to malic enzymes from other bacteria. A defined *malE* mutant grew as well as the parental strain on either glucose or acetate minimal medium, however, showed lower growth rates on lactate medium [117]. These results led to the hypothesis that malic enzyme might be involved in the generation of NADPH on substrates known to have a low (or no) flux through the pentose pathway. Another hypothesis is that malic enzyme, together with pyruvate carboxylase and the NADH-dependent malate dehydrogenase, would catalyze an ATP-dependent metabolic cycle generating NADPH from NADH without the loss of carbon [237] as depicted in Fig. 2. While there is yet no experimental evidence for the operation of such a cycle in *C. glutamicum*, it might be advantageous for the cells in view of the obvious absence of any gene similar to those demonstrated as encoding transhydrogenase activity.

Very recently, Netzer et al. [266] found that overexpression of the *malE* gene and thus, high malic enzyme activity allowed the growth of a pyruvate kinase mutant of *C. glutamicum* on gluconeogenic substrates, such as acetate or citrate. The authors explained the complementation by an increased supply with pyruvate in the *malE* overexpressing strain and speculated that the regulation of the *malE* expression in the wild type of *C. glutamicum* precludes a role of malic enzyme for pyruvate generation under gluconeogenic conditions.

Oxaloacetate decarboxylase activity has been detected in several *C. glutamicum* strains [236] and Jetten and Sinskey [138] were able to purify and biochemically characterize an oxaloacetate decarboxylase protein from one of the strains. The molecular mass of the subunits (32 kDa) and of the native enzyme (118 kDa) suggested an α4 subunit structure. It is highly specific for oxaloacetate and inhibited by several effectors (Table 4). Due to the fact that the purified enzyme did not require sodium ions for its activity and was not inhibited by avidin, it does not belong to the membrane-bound sodium-dependent oxaloacetate decarboxylases. Instead, it was dependent on divalent cations and accordingly falls into the class of divalent-cation-dependent oxaloacetate decarboxylases.

Although the enzyme has been thoroughly characterized, the *C. glutamicum* gene encoding oxaloacetate decarboxylase has not been identified so far. Since also metabolic network analysis did not identify direct carbon fluxes from oxaloacetate to pyruvate [10], the function and role of oxaloacetate decarboxylase for growth and amino acid production remains unclear.

5.3. Carbon fluxes and regulation at the corynebacterial PEP–pyruvate–oxaloacetate node

The surprising diversity of the enzymes at the PEP–pyruvate–oxaloacetate node in *C. glutamicum* (Fig. 7)

and the fact that all of them are present with significant specific activities in extracts of cells grown on glucose provokes the questions for the actual in vivo fluxes and flux ratios at this metabolic branch point. Genetic and enzymatic studies are inadequate for answering this question and therefore, sophisticated and extensive metabolic flux analysis techniques have been applied [257,267–273].

The two major routes of carbon flux at the corynebacterial PEP–pyruvate–oxaloacetate node are the C3-carboxylation and the oxidative decarboxylation of pyruvate, i.e. anaplerosis and the fueling of the TCA cycle. These carbon fluxes and also the flux partition ratios vary significantly. However, from comparative analyses with isogenic strains under different conditions [274,275] and from flux genealogy of lysine-producing *C. glutamicum* strains [276], it became evident that an increased carbon flux into the lysine biosynthetic pathway is always accompanied both by an increase in anaplerosis and a decrease in the flux towards the TCA cycle via acetyl-CoA. In glutamate-producing cells of *C. glutamicum*, the anaplerotic flux was also increased whereas the flux into the TCA cycle via the pyruvate dehydrogenase complex was unchanged when compared to exponentially growing cells [275].

Labelling experiments with *C. glutamicum* using ^{13}C-glucose with subsequent nuclear magnetic resonance analyses or mass spectrometry in combination with metabolite balancing repeatedly revealed that in addition to the C3-carboxylation activity (forward flux), in vivo there is a strong C4-decarboxylating activity (backward flux), which varies significantly under different growth and/or production conditions (Table 5; [272,274–279]). The net anaplerotic activity in fact represents the sum of bidirectional interconversion of C3- and C4-metabolites by simultaneous carboxylation and decarboxylation reactions. The cyclic flux can be threefold in excess over the anaplerotic flux [10,279] and from the data given in Table 5 it becomes clear that the increased anaplerotic flux during lysine and glutamate production is due to a severe decrease of the C4-decarboxylation. As outlined in the previous chapters, bidirectional C3–C4 interconversions have also been observed in *B. subtilis* [221,280] and *E. coli* [11,194], in particular under glucose limitation.

The individual in vivo fluxes (forward, back and parallel) at the PEP–pyruvate–oxaloacetate node of *C. glutamicum* recently were precisely quantified in a single experiment [10]. The results indicated that, although the in vitro specific activity of pyruvate carboxylase is much lower than that of PEP carboxylase (0.02 U/mg protein vs. 0.15 U/mg protein) [77,234], the pyruvate carboxylase reaction constitutes the principal anaplerotic route (Fig. 8). PEP carboxylase operates in a parallel sense with a small but significant contribution of about 10%. The results additionally indicated that the

Table 5
PEP-/pyruvate- (C3-) carboxylation fluxes, malate-/oxaloacetate- (C4-) decarboxylation fluxes and anaplerotic net fluxes in *C. glutamicum* under different conditions as determined by ^{13}C NMR analyses

C. glutamicum strain	Cultivation condition[a]	C3-carboxylation [%]	C4-decarboxylation (%)	Anaplerotic net flux (%)	Reference
	Batch culture				
Wild-type (ATCC13032)	Glucose	89	69	20	[279]
Wild-type (ATCC13032)	Glucose + acetate	85	106	−21[b]	[279]
	Continuous culture				
MH20-22B[c]	Lysine-producing	68	30	38	[278]
LE 4[d]	No production	96	72	24	[275]
LE4	Glutamate-producing	47	18	29	[275]
MH20-22BΔ*gdh* (pEKgdh), high NADH-dependent GDH[e] activity	lysine-producing	54	10	44	[277]
MH20-22BΔ*gdh* (pEKExpgdh), high NADPH-dependent GDH[e] activity	lysine-producing	65	29	36	[277]

The net anaplerotic fluxes represent the sum of the C3-carboxylation and the C4-decarboxylation fluxes. Data are given as percentage of the molar glucose uptake rate.
[a] All in minimal medium with glucose. Additional carbon sources are specified.
[b] Negative values in fact represent fluxes towards gluconeogenesis.
[c] Lysine producer.
[d] MH20-22b with wild-type aspartokinase.
[e] GDH, glutamate dehydrogenase.

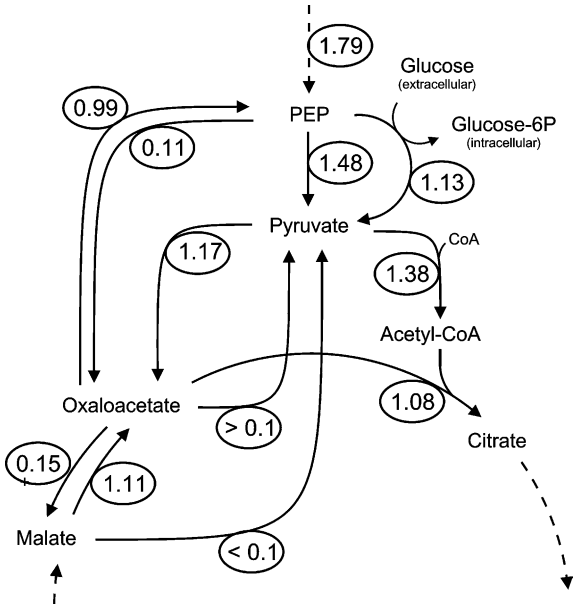

Fig. 8. Distribution of metabolic fluxes at the PEP–pyruvate–oxaloacetate node in *C. glutamicum*. All fluxes are given in mmol/g dry weight/h and were obtained from carbon-limited continuous cultures at a growth rate of 0.1 h^{-1} [10].

C4-decarboxylating backward flux at the PEP–pyruvate–oxaloacetate node is more or less exclusively based on PEP carboxykinase activity. As the authors did not find a significant carbon flux from oxaloacetate or malate to pyruvate, neither malic enzyme nor oxaloacetate decarboxylase were involved in the C4–C3 interconversions. However, it becomes clear that under glycolytic conditions, pyruvate carboxylase, PEP carboxykinase and pyruvate kinase are responsible for an energy (ATP/GTP) consuming (futile) cycle in which pyruvate is carboxylated to oxaloacetate, oxaloacetate is decarboxylated to PEP, and PEP is converted to pyruvate again (see Fig. 7). Although the physiological function of this cycling remains unknown, the results of Petersen et al. [10] suggest that the PEP carboxykinase may have physiological significance under non-gluconeogenic conditions. This conclusion has been substantiated by the findings that different PEP carboxykinase activities have impact on the intracellular fluxes and on the intracellular concentrations of key intermediates (such as oxaloacetate, 2-oxoglutarate and pyruvate) involved in the regulation of the enzymes at the PEP–pyruvate–oxaloacetate node [248]. However, the physiological function and the advantage of the excessive cycling under the different conditions and also the physiological function of PEP carboxykinase under glycolytic conditions are far from elucidated and remain to be clarified.

Using stable isotopes and mass spectrometry, Klapa et al. [272] also resolved the anaplerotic fluxes of a different strain of *C. glutamicum* in glucose-limitated continuous culture (growth rate of 0.1 h^{-1}). These authors also found a high cyclic flux between C3- and C4-intermediates at the PEP–pyruvate–oxaloacetate node, however, their results indicated a very high exchange flux between exclusively pyruvate and oxaloacetate/malate, no flux via PEP carboxykinase and a high flux from oxaloacetate/malate to pyruvate, due to oxaloacetate decarboxylase and/or malic enzyme. These results support the idea of futile cycling between the C3- and C4-intermediates, but clearly contradict the flux estimates of Petersen et al. [10,248]. Klapa et al. [272] speculated that the different (high) PEP carboxykinase flux observed by Petersen et al. might be due to use of different strains or to the use of lactate as a second labelling source. Lactate, although used in small quantities, may have triggered gluconeogenesis and hence, high PEP carboxykinase activity.

From the data mentioned above it obvious that the carbon flux at the PEP–pyruvate–oxaloacetate node in *C. glutamicum* is different under various conditions and that it is regulated by mechanisms at enzyme activity and gene expression levels. However, the question for appropriate and coordinated regulation of the *C. glutamicum* enzymes and genes of anaplerosis and other central metabolic pathways in response to a given condition arises. A coordinated regulation of the anaplerotic enzymes and of the TCA cycle enzymes can be expected to provide a balanced energy and precursor generation for growth under a given condition. In fact, recent transcriptome and proteome analyses with the wild-type of *C. glutamicum* grown on either glucose- or on acetate-medium revealed coordinated and specific expression of several genes coding for enzymes of the PEP–pyruvate–oxaloacetate node (pyruvate kinase, pyruvate dehydrogenase subunit E1, PEP carboxykinase, malic enzyme) and for enzymes of the TCA cycle (citrate synthase, aconitase, succinate dehydrogenase) [265,281,282]. All these genes obviously are under transcriptional control in response to the presence or absence of acetate in the growth medium. Due to the identification of a highly conserved 13-bp motif (AAAACTTTGCAAA) in the upstream region of some of the genes mentioned above [283], the authors speculated about induction or repression of these genes by (a) common regulatory device(s). Moreover, the authors speculated that the 13-bp motif has broader significance in a global regulatory system controlling the central metabolic pathways in *C. glutamicum* in response to the substrate present in the growth medium.

5.4. Metabolic engineering of the node for amino acid production

Nowadays *C. glutamicum* is employed worldwide for the large-scale biotechnological production of most amino acids. A variety of amino acids directly or indirectly originate from the PEP–pyruvate–oxaloacetate node and therefore, the enzymes at this node, in partic-

Table 6
Enzyme activities and amino acid production by recombinant strains of *C. glutamicum* wild-type (WT) and of the lysine producers DG52-5 or MH20-22B

C. glutamicum strain	Relevant enzyme[a]	Spec. activity of relevant enzyme (mU/mg protein)[b]	Glutamate concentration (mM) in the culture fluid[c]	Lysine concentration (mM) in the culture fluid[d]
WT	PCx	20	8	n.d.[e]
WTΔpyc	PCx	<1	3	n.d.[e]
WT(pVWEx-pyc)	PCx	202	54	n.d.[e]
DG52-5	PCx	8	n.d.[e]	34
DG52-5Δpyc	PCx	<1	n.d.[e]	14
DG52-5(pVWEx-pyc)	PCx	88	n.d.[e]	50
WT	PEPCk	44	10	n.d.[e]
WTΔpck	PEPCk	<3	44	n.d.[e]
WT(pEK-pckB)	PEPCk	413	4	n.d.[e]
MH20-22B	PEPCk	75	n.d.[e]	54
MH20-22BΔpck	PEPCk	<3	n.d.[e]	66
MH20-22B(pEK-pckB)	PEPCk	680	n.d.[e]	43

Data were taken from Peters-Wendisch et al. [249] and from Riedel et al. [84].
[a] For enzyme abbreviations see Fig. 1.
[b] Determined in extracts of cells grown on minimal medium containing glucose.
[c] Glutamate production was induced by the addition of Tween 60 and determined after 24 h of cultivation in minimal medium glucose.
[d] Lysine was determined after 48 h of cultivation in minimal medium glucose.
[e] n.d., not determined.

ular the anaplerotic enzymes, have been regarded as fascinating targets for metabolic engineering of *C. glutamicum* (e.g. [257,267,273]). Menkel et al. [284] already showed that addition of fumarate to the growth medium and thus increasing the oxaloacetate and aspartate availability led to an about 30% higher lysine yield with a producer strain. From this result it was concluded that the supply of the precursors oxaloacetate or aspartate is rate limiting for optimal lysine production. As outlined above, PEP carboxylase has long been considered as a prime target for the molecular breeding of hyperproducing strains [257]. However, analysis of strains with altered activity of PEP carboxylase [251,252,255,285], showed that this enzyme has only a minor impact on amino acid (over)production. The identification of the pyruvate carboxylase as alternative anaplerotic enzyme [77,80] as well as quantitative in vivo flux determinations at the PEP–pyruvate–oxaloacetate node [10] substantiated this conclusion and drew major attention to this enzyme. In fact, overexpression and thus increasing the pyruvate carboxylase activity resulted in higher accumulation of TCA cycle-derived amino acids (glutamate, lysine or threonine) in culture supernatants of the respective strains (Table 6) [249]. In contrast, pyruvate carboxylase-deficient *C. glutamicum* producer strains showed significantly lower amino acid formation. In accordance with these results, Ohnishi et al. [263] obtained a significant increase in lysine accumulation when they introduced a mutated pyruvate carboxylase gene (probably coding for a feedback-inhibition-resistant pyruvate carboxylase) by allelic exchange into a lysine-producing strain. All these results unequivocally identified the pyruvate carboxylase reaction as a major bottleneck for amino acid production.

Another crucial reaction for the production of amino acids derived from the TCA cycle is the PEP carboxykinase reaction. In contrast to the situation found with pyruvate carboxylase, abolition of PEP carboxykinase activity led to an increase of glutamate (440%) and lysine (120%) production wheras increasing the PEP carboxykinase activity led to significantly reduced productivity (40% and 20%, respectively) (Table 6) [84]. Having in mind the bidirectional carbon fluxes between oxaloacetate and PEP and taking into account the results of the comparative carbon flux analysis and intracellular metabolite quantification in the wild-type and in derivatives with altered PEP carboxykinase activities [248] the positive effect of decreased PEP carboxykinase activity on the production of glutamate and lysine can be explained just by an increase of the net carbon flux towards oxaloacetate and an increase of the intracellular oxaloacetate concentration and thus by an increase of precursor supply.

6. Concluding remarks

The biochemistry, physiology, and molecular biology of the enzymes of the PEP–pyruvate–oxaloacetate node of several bacteria have been studied intensively and a substantial amount of knowledge has been accumulated. The molecular and functional studies of many of the enzymes and genes involved in the pathways allowed to identify the peculiarities of the single steps of the node and to partly elucidate the regulation of enzyme activities and control of gene expression. Disruption and overexpression of some of the genes and analysis of the recombinant bacteria clarified the relevance of the

respective enzyme activity for growth under a given condition and in some cases, for primary metabolite production. Due to quantitative assessment of metabolic fluxes through the central metabolism and of the flux distributions at metabolic branch points, detailed information is not only available for the single reactions within the node of a given organism, but also for the pathways leading to or away from the PEP–pyruvate–oxaloacetate node of a variety of microorganisms.

Reviewing the newer studies on the PEP–pyruvate–oxaloacetate node, it becomes evident that some of the enzymes, aside from their classically recognized functions in catabolism, anaplerosis and gluconeogenesis, play further roles in the metabolism of some bacteria. One example is simultaneous operation of glycolytic and gluconeogenic enzyme pairs in ATP-dissipating futile cycles under several conditions [10,144,190,221]. Although certainly increasing the flexibility of the PEP–pyruvate–oxaloacetate node, the associated energetic burden can be expected to be detrimental to high-level metabolite production. A rather prominent example is the PEP carboxykinase. In addition to its generally recognized gluconeogenic formation of PEP from oxaloacetate, it has recently been demonstrated to contribute to catabolism and anaplerosis in *E. coli*, when operating in combination with the glyoxylate shunt [11], and in *B. subtilis* and other bacteria, when operating in the reverse direction [83,222], respectively. Notably, even in wild-type strains, the PEP–pyruvate–oxaloacetate node is not necessarily optimally organized for growth under a given condition, as was convincingly shown for rapid growth of *E. coli* on glucose [161–163] or on gluconeogenic substrates [145].

Despite the accumulated information on the PEP–pyruvate–oxaloacetate node in bacteria, significant gaps remain in our knowledge of the characteristics, regulation, and relevance of some of the enzymes and of the overall control of the carbon flow at the node. The influence of allosteric regulation of some of the enzymes on the flux through the node has only scarcely been investigated, and quantitative understanding on which regulator controls in vivo carbon flow is lacking completely. Moreover, the physiological significance of the known side activities of some of the enzymes is not clear and the molecular mechanisms of transcriptional and post-transcriptional regulation that governs expression of the genes for the enzymes are not yet fully elucidated. The phosphoproteome of *C. glutamicum* has recently been investigated and pyruvate carboxylase and pyruvate kinase were identified as phospho-proteins [286]. A challenge of future studies will be the localization of the phosphorylation sites within the enzymes and elucidation of the regulatory relevance of phosphorylation/dephosphorylation. For a comprehensive understanding of the PEP–pyruvate–oxaloacetate node as the central switch point between the major metabolic pathways in bacteria and to optimize its operation for biotechnological processes, these issues have to be addressed in the future.

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