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REVIEW

Minimizing acetate formation in *E. coli* fermentations

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Abstract *Escherichia coli* remains the best-established production organism in industrial biotechnology. However, when aerobic fermentation runs at high growth rates, considerable amounts of acetate are accumulated as by-product. This by-product has negative effects on growth and protein production. Over the last 20 years, substantial research efforts have been expended on reducing acetate accumulation during aerobic growth of *E. coli* on glucose. From the onset it was clear that this quest would not be a simple or uncomplicated one. Simple deletion of the acetate pathway reduced the acetate accumulation, but other by-products were formed. This mini review gives a clear outline of these research efforts and their outcome, including bioprocess level approaches and genetic approaches. Recently, the latter seems to have some promising results.

Keywords Acetate reduction · *Escherichia coli* · Metabolic engineering

Introduction

Escherichia coli was the first and is still one of the most commonly used production organisms in industrial biotechnology. Aerobic high cell density cultures of *E. coli* are most frequently used to arrive at high biomass yields and high metabolite/protein concentrations. Normally, glucose is fed as a carbon source in these high cell density fed-batch cultures. Glucose is a cheap and simple molecule, which enters glycolysis (flow from glucose to acetyl CoA) and the central metabolism through a minimum of steps. Furthermore, in a medium with several carbon sources, glucose is preferred as a result of catabolite repression. Glycolysis is the trunk route of intermediary sugar metabolism in enteric bacteria, which canalizes 72% of the carbon supply. However, during aerobic fermentation runs at high growth rates, considerable amounts of acetate are accumulated, as described by Akkeson et al. [1] and references therein. Not only is acetate production a loss of carbon and therefore an economic sink, it is also detrimental to recombinant protein production and inhibits cell growth [2]. For more information on overcoming acetate in *E. coli* recombinant protein fermentations see reference [3].

Formation of acetate in *E. coli* cultures under fully aerobic conditions can be caused by two phenomena. On the one hand, a (local) lack of dissolved oxygen activates the fermentation pathways causing acetate excretion. This is referred to as mixed-acid fermentation. On the other hand, this acetate excretion is also due to a metabolic overflow mechanism, caused by an imbalance between the rapid uptake of glucose and its conversion into biomass and products, diverting acetyl-CoA from the TCA-cycle toward acetate [1].

The two major aerobically active acetate-producing pathways in *E. coli* are pyruvate oxidase (*poxB*) and acetate kinase/phosphotransacetylase (*ackA-pta*). Two enzymes comprise the *ackA-pta* pathway: phosphotransacetylase [EC 2.3.1.8] reversibly converts acetyl-CoA and inorganic phosphate to acetyl phosphate and CoA, and acetate kinase [EC 2.7.2.1] reversibly converts acetyl phosphate and ADP into acetate and ATP [4]. The two genes include one

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operon [5] and are considered to be important for balanced carbon flux within the cell during exponential growth, both aerobically and anaerobically [6, 7]. *E. coli* uses the *ackA-pta* pathway as an ATP production source under anaerobic and even aerobic conditions [7].

The second enzyme, pyruvate oxidase [EC 1.2.2.2], is a pheripheral membrane protein that converts pyruvate, ubiquinone and H_2O to acetate, ubiquinol and CO_2. It has been reported to be a nonessential aerobic enzyme active in the early stationary phase [8, 9]. However, more recent studies have shown that pyruvate oxidase plays an important role in the aerobic growth efficiency of *E. coli* [10], perhaps to preserve the pool of free CoA-SH. Dittrich et al. [2] confirm that the *poxB* pathway is more active during the late exponential and stationary phases, whereas the *ackA-pta* pathway is more active in the exponential stage of the cell growth. They also report that the two acetate-producing pathways are affected by culture conditions such as pH. Acidic environments repress the *ackA-pta* pathway, but activate the poxB pathway. In addition, acetate itself has a strong negative effect on the two pathways.

Acetate formation has several disadvantages:

- acetate concentrations above ca. 1 g/l are damaging for both the biomass production and the production of recombinant proteins [11]
- besides the inhibition on recombinant protein production, acetate has a negative effect on the stability of intracellular proteins [12]
- organic acids already show negative effects at concentrations much lower than those for mineral acids. The non-dissociated form of acetate can move freely through the cell membrane and thus accumulates in the medium. A part of this extracellular, non-dissociated form will re-enter the cell and dissociate due to the higher internal pH. Acetate thus acts as a proton conductor and the process causes a reduction in proton motive force [13]
- accumulation of acetate in the medium will acidify the medium. When the pH is below 5.0, cell lysis will appear due to the irreversible denaturation of proteins and DNA [14].

The level of acetate produced during aerobic fermentations is depending on the *E. coli* strain, the growth conditions, the actual glucose concentration in the medium and the overall composition of the fermentation medium. For more detailed information about why, when and how bacterial cells excrete acetate, see Ref. [15].

Researchers have tried a wide variety of strategies to reduce acetate accumulation in high cell density fed-batch *E. coli* fermentations. These strategies are situated at two levels: the bioprocess level and the genetic level.

Bioprocess level approaches to minimize acetate formation

These methods mostly intervene in the medium composition and/or the cultivation conditions. The fermentation run can be optimized by controlling a range of parameters such as temperature, agitation regime, volume, foaming, dissolved oxygen tension (DOT), pH, optical density, (limiting) substrate concentration, etc.

A culture of *E. coli* will generate acetate when the cells surpass a threshold-specific rate of glucose consumption, regardless of the availability of oxygen to the culture [3]. In the literature, several specific fermentation strategies to reduce acetate production levels are mentioned [1, 16–20]. These methods are based on mathematical models that describe growth patterns and the expected demand for nutrients. These strategies include various glucose-feeding approaches [21–27], limitations of growth rate by substrate-limited fed batch schemes [16, 18, 26, 28–30] and utilization of alternative feeds such as glycerol [26, 31], mannose [32] or fructose [33]. For example, reduced acetate and an increase in protein yield have been reported when fructose was used as carbon source instead of glucose [33]. Also supplementing the medium has proven to be positive on reducing acetate [34]. Recently, the combination of glucose pulses with an amino-acid containing feed stream has been demonstrated to be successful to minimize acetate production [35]. Another approach to hold the growth rate below the threshold for acetate production is the pH-stat, where a nutrient feed is activated if the pH increases and variants, where the culture is dosed with more nutrient than necessary. However a fundamental drawback of the pH-stat is that it detects starvation rather than the acetate threshold directly [3]. In general, a consequence of limiting the growth rate is that biomass is generated at a slower rate than the cells are capable of achieving.

Instead of lowering the growth rate, the produced acetate can be removed from the culture during the fermentation process to reduce the inhibitory effects of acetate. In literature, the use of dialysis-fermentors is reported to remove acetate from the culture [36, 37]. Dialysis is defined as the separation of solute molecules by their unequal diffusion through a semi-permeable membrane based on a concentration gradient. Recently, another method to remove acetate from the fermentor was reported via the use of macroporous ion-exchange resins [38]. However, methods to remove acetate from the culture tend to remove also nutrients. In addition, this strategy does not deal with the fact that carbon is diverted to a by-product and thus the economic sink.

Although, these methods are extensively used in the industry, they are not the best solutions because they

undermine maximum growth and production capacity. Therefore, we will emphasize genetic approaches to minimize acetate formation.

Genetic approaches to minimize acetate formation

Several strategies, which intervene with acetate formation on the genetic level have also been reported. These strategies are based on the alteration of the central metabolism of *E. coli* (see Fig. 1, Table 1). First, the effect of alterations in the glucose uptake mechanism and in the TCA cycle will be discussed. The pathway from glycolysis to the TCA cycle is very important because of the many reactions, which play a role in the pyruvate branch point. From this branch point, the carbon flux can be directed to acetate production via the genes coding for acetate kinase (*ackA*), phosphotransacetylase (*pta*), acetyl-CoA synthase (*acs*) and pyruvate oxidase (*poxB*). The flux can also be directed to the TCA-cycle (citrate synthase, *gltA*) where the glyoxylate bypass plays an important regulation control (isocitrate lyase, *ackA*; malate synthase, *aceB* and isocitrate dehydrogenase; *icd*). In the final paragraph, the influence of alterations in coenzyme pools on the acetate metabolism will be discussed.

Since *E. coli* is a facultative anaerobic strain, part of the glucose (even under aerobic conditions) will be catabolized via fermentation (consuming no oxygen). Besides a lower energy yield per mol glucose obtained by anaerobic fermentation, this causes a faster utilization of glucose by the cells, as compared with aerobic respiration [39]. Because of the occurrence of reactions which run normally anaerobically in aerobic conditions, some enzymes which are active under anaerobic growth conditions will be discussed. Strictly anaerobic culture strategies and strategies based on involvement of non-*E. coli* pathways (e.g. *pyc* pathway) will not be discussed.

The phosphoenolpyruvate (PEP):carbohydrate phosphotransferase systems (PTSs)

The PEP:carbohydrate phosphotransferase systems, which are both transport and sensing systems, are an example of

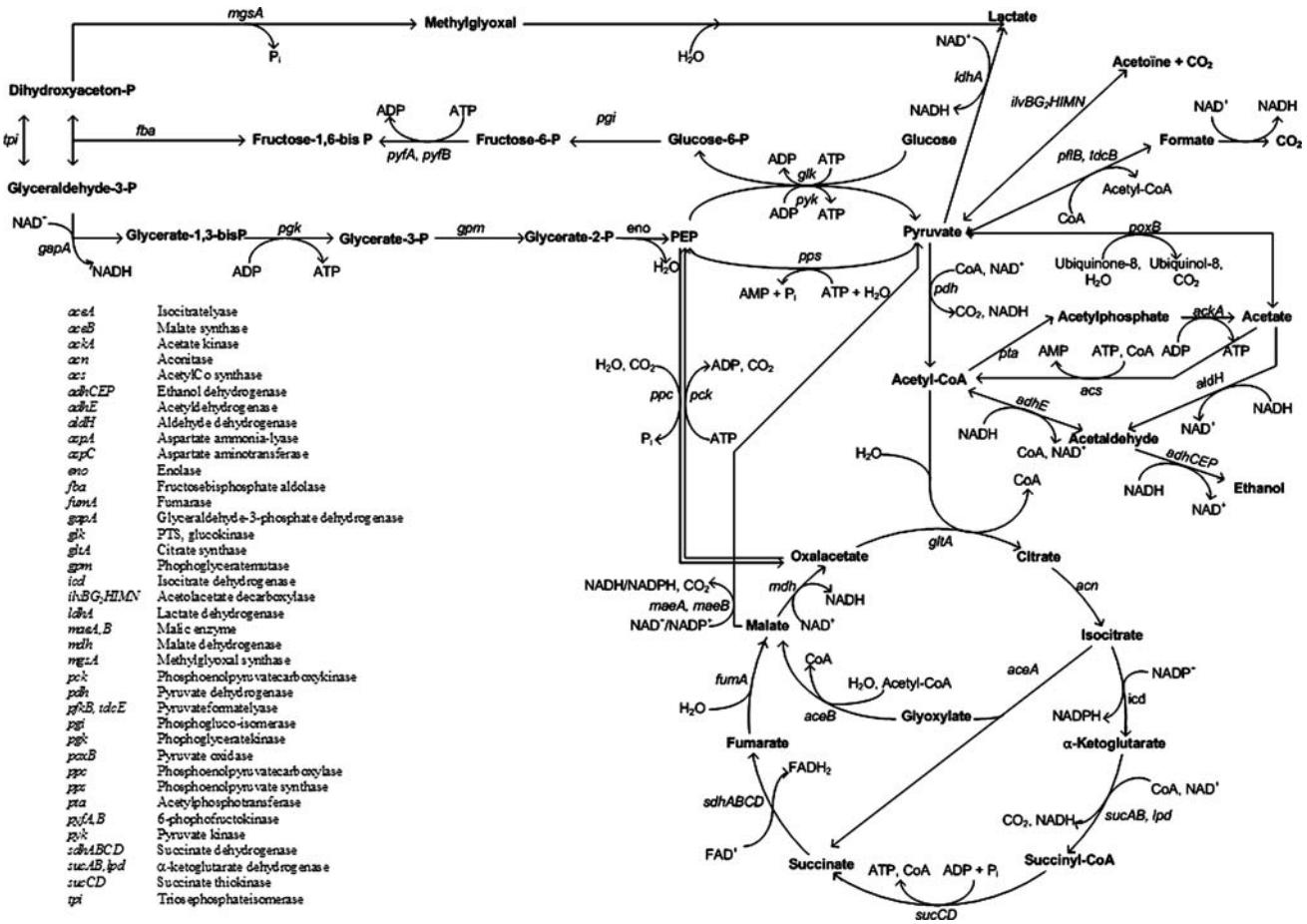

Fig. 1 The central metabolism of *Escherichia coli*

Table 1 Influence of genetic approaches to minimize acetate formation

Pathway	Gene	Protein	KO/OE	Result	Reference
PTS	*ptsG*	Glucose specific enzyme II	KO	Glycolysis flux ↓, no acetate excretion	[40]
				Energy metabolism ↓	[17]
				Acetate ↓	[43]
				Growth rate ↓, flux to TCA cycle ↑	[42]
				Acetate ↓, recombinant protein ↑, biomass ↑	[41]
	arcA	Regulator *ptsG*	KO	ptsG ↑	[44]
			OE	Glucose consumption ↓	[44]
				Acetate accumulation ↓	
			KO *ptsG*, *pykF* and *pykA*	Acetate ↓	[43, 91]
Glycolysis	*pfk*	Phophofructokinase	OE	Ethanol ↓, lactate and acetate ↑	[92]
Pyruvate branchpoint	*pyk*	Pyruvate kinase	KO	KO_1: growth rate and acetate ↓	[50]
				KO_2: growth rate and acetate ↓↓	[51]
			KO *PykF*		[53]
	pdh	Pyruvate dehydrogenase	Inhibition PDH	No acetate production, growrh rate ↓, lactate ↑	[93]
	pfl	Pyruvate formate lyase	KO	Lactate ↑, small ↓ flux acetyl CoA to acetate	[94–96]
	ldh	Lactate dehydrogenase	OE	Acetate ↓	[97]
			KO *pfl* and *ldh*	Acetate and lactate ↓, malate ↑	[98]
	ppc	PEP carboxylase	KO	Acetate ↓, growth rate ↓, glyoxylate shunt ↑, glycolysis and PPpw ↓	[60, 62, 63]
			OE	No/↓ acetate, growth yield ↑,	[17, 59–61]
	pck	PEP carboxykinase	KO	PEP carboxylation ↓, glyoxylate ↑, no acetate on high glucose	[57]
			OE	Acetate ↑	[59]
			OE *ppc* and *pck*	Fermentative products ↑,growth yield ↓	[58]
			OE *ppc* or *pck*	PPC bypass:Krebscycle ↓↓	[55, 57]
	ackA	Acetate kinase	KO *ackA* or *pta*	Acetate ↓↓, groxth rate ↓, formate and lactate ↑	[2, 11, 54, 97, 99]
	pta	Phosphotransacetylase			
	acs	Acetyl-CoA synthetase	KO	No clear conclusions	[11]
			OE	Acetate ↓↓	[68]
	poxB	Pyruvate oxidase	KO	C-yield ↓↓	[10]
	gltA	Citrate synthase	KO	Acetate ↑↑, pyruvate and formate ↑	[72]
			OE	Acetate ↓	[60]
	aceA	Isocitrate lyase	Stimulation shunt	Acetate ↓	[17]
	aceB	Malate synthase			
	icd	Isocitrate dehydrogenase	KO	Accumulation citrate	[73]
			OE	No rate limiting step Krebs	[74]

Abbreviations: *KO* knock-out, *OE* overexpression

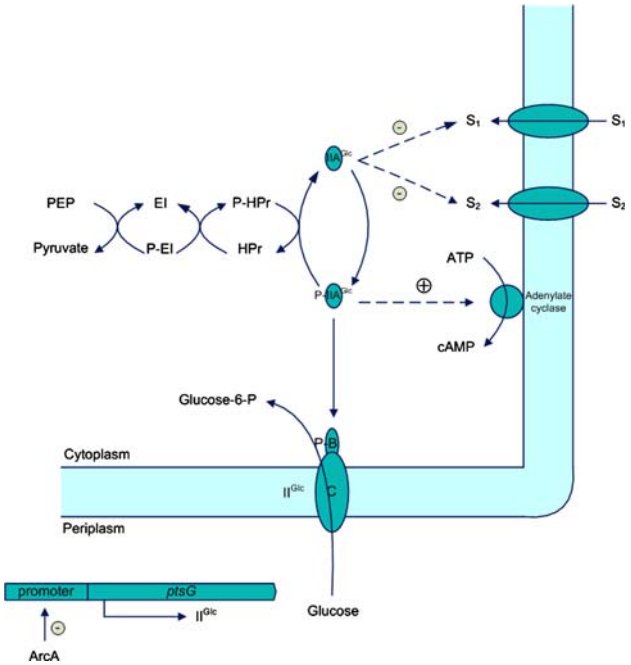

Fig. 2 PTS-system and regulation

group translocation enzymes. PTS (Fig. 2) is involved in the transport and the phosphorylation of a large number of carbohydrates (PTS carbohydrates), in the movement of cells toward these carbon sources (chemotaxis), and in the regulation of a number of metabolic pathways. The PTS catalyzes the following overall process:

$$\mathrm{PEP}_{(\mathrm{in})} + \mathrm{carbohydrate}_{(\mathrm{out})} \xrightarrow{\mathrm{PTS}} \mathrm{pyruvate}_{(\mathrm{in})} + \mathrm{carbohydrate} - P_{(\mathrm{in})}$$

Carbohydrate phosphorylation is coupled to carbohydrate translocation across the cytoplasmic membrane, the energy for these processes being provided by the glycolytic intermediate PEP. PTS consists of three kinds of proteins: enzyme I and histidine protein (HPr), which participate in the phosphorylation of all PTS carbohydrates and thus have been called the general PTS proteins, and enzyme II, which is carbohydrate specific.

Chou et al. [40], tried to reduce the acetate excretion by knocking out the *ptsG* gene, coding for the glucose specific enzyme II of PTS [EC 2.7.1.69]. This method did not totally prevent the uptake of glucose, but reduced its uptake rate. Consequently, the flux through the glycolysis was decreased, causing a reduced acetyl-CoA accumulation. Chou et al. [40] observed no acetate excretion in cultures of this mutant. Similar results were found in [41–43]. Moreover, Han et al. [41] found an increase in biomass and recombinant protein production as a result of knocking out *ptsG*. Another way to intervene in the PTS is to influence the regulation of *ptsG*. It was found that the regulator ArcA binds to the promoter of *ptsG*. Deletion of the *arcA* gene caused about a twofold increase in the *ptsG* expression. Overexpression of *arcA* significantly decreased glucose consumption and hence decreased the acetate accumulation [44]. Knocking out *ptsG* and overexpressing *arcA*, however, aregenetic variants of limiting the glucose feed rate. Moreover, mutation of a PTS gene causes an efficiency reduction in the energy metabolism [17].

However, recently, it is been reported that the deletion of the ArcA gene in combination with the overexpression of a heterologous NADH oxidase increased the glycolytic flux and reduced acetate production [45, 46].

Pyruvate branch point

Many strategies of metabolic engineering are focusing on the enzymes around the pyruvate branch point since the intracellular level of pyruvate has an immediate influence on acetate excretion. Pyruvate is the substrate or end product of many reactions and thus an interesting target for manipulation. The enzymes of the main reactions around pyruvate are: pyruvate kinase (*pyk*), pyruvate dehydrogenase (*pdh*), pyruvate formate lyase (*pfl*), lactate dehydrogenase (*ldh*), PEP-carboxylase (*ppc*) and PEP-carboxykinase (*pck*).

Lowering the pyruvate pool has been many times described in the literature as a way to reduce acetate production [47, 48].

Pyruvate kinase (pyk)

Pyruvate kinase [PYK, EC 2.7.1.40] is one of the key enzymes of glycolysis. It catalyzes the conversion of PEP into pyruvate and simultaneously converts ADP to ATP:

$$\mathrm{PEP} + \mathrm{ADP} \xrightarrow{\mathrm{PYK}} \mathrm{pyruvate} + \mathrm{ATP}$$

In most cell types, the flux through this reaction controls the global flux through glycolysis. There are two isoenzymes of pyruvate kinase, encoded by *pyk-I* (or *pyk-F*) and *pyk-II* (or *pyk-A*). The enzyme PYK-F is activated by fructose-1,6-bisphosphate, while PYK-A is activated by intermediary products of the pentose phosphate pathway such as ribose-5-phosphate [49].

Inactivation of one or both pyruvate kinase isoenzymes has already been tried several times to reduce acetate production [50–52]. Several studies have reported that glycolysis was down regulated in *E. coli pykF* mutants under aerobic conditions [50, 53]. It was found that the flux through phosphoenol pyruvate carboxylase and malic enzyme were up-regulated in the $pykF^{-}$ mutant as

compared with the wild type, and acetate formation was significantly reduced in the mutant. Inactivation of one *pyk*-enzyme caused a slight decrease of the maximum growth rate. This indicates that the other *pyk*-enzyme can compensate for the supply of the pyruvate pool, together with the PTS. Inactivation of both *pyk*-enzymes causes a major decrease in the growth rate and the acetate production [51]. Emmerling et al. [50] reported that relatively more oxaloacetate is derived from PEP and more pyruvate from malate in comparison with the wild type. This mechanism is probably activated to compensate to some extent for the pyruvate kinase knockout.

Pyruvate dehydrogenase (pdh), expressed under aerobic culture conditions, in E. coli

Pyruvate dehydrogenase [PDH, EC 1.2.4.1] complex catalyzes the conversion of pyruvate into acetyl-CoA with the formation of one molecule CO_2.

$$\text{pyruvate} + \text{CoA - SH} + \text{NAD}^+ \xrightarrow{\text{PDH}} \text{acetyl - CoA} + \text{NADH} + \text{H}^+ + \text{CO}_2$$

A too-large pool of acetyl-CoA contributes to a large extent to the overflow of acetate. Elmansi and Holms [54] tried to reduce the acetate excretion by decreasing the flux from pyruvate to acetyl-CoA. This was achieved by adding 3-bromopyruvate, an inhibitor, which directly acts on the active domain of pyruvate dehydrogenase. They reported no acetate production at all. However, lactate was still produced and a decrease of the growth rate with respect to the wild type was observed.

PEP carboxylase (ppc) and PEP carboxykinase (pck)

PEP carboxylase [PPC, EC 4.1.1.31] converts PEP into oxaloacetate and is referred as PPC shunt by Noronha et al. [55]:

$$\text{PEP} + \text{CO}_2 \xrightarrow{\text{PPC}} \text{oxaloacetate} + P_i.$$

This reaction is activated by acetyl-CoA, guanosinetriphosphate and fructose-1,6-biphosphate, and inhibited by aspartate and malate [56].

PEP-carboxykinase [PCK, EC 4.1.1.49] catalyzes the reverse reaction with consumption of 1 molecule ATP:

$$\text{oxaloacetate} + \text{ATP} \xrightarrow{\text{PCK}} \text{PEP} + \text{ADP} + \text{CO}_2$$

PCK is inhibited by high levels of PEP and nucleotides [56]. The purpose of PCK is to maintain the PEP:oxaloacetate ratio and stabilize the pool of intermediate products of the Krebs cycle. The net reaction of the cycle formed by both reactions consumes one molecule of ATP. In *E. coli* wild type strains, this futile cycle is strongly regulated. Inactivation of the *pck* gene causes a decrease of PEP carboxylation and a stimulation of the glyoxylate cycle. Yang et al. [57] reported that *pck* deletion mutants are able to grow on high concentrations of glucose without acetate production.

Chao and Liao [58] decided that overexpression of both enzymes gives an increased activation of the futile cycle with a higher production of fermentative products as a consequence. The double overexpression was also responsible for less growth and a higher consumption of glucose and oxygen. All these are consequences of the leakage of energy via the futile cycle.

Simple overexpression of the *pck* causes a slight increase in acetate production [59]. On the other hand, overexpression of *ppc* can completely eliminate acetate production [59, 60]. According to Chao and Liao [59], overexpression of *ppc* decreases the glucose consumption rate and organic acid excretion, while growth and respiration rate remain unchanged. Farmer [17] described the effect of overexpressing PPC in *E. coli* VJS632 aerobic cultures and concluded that the final acetate concentration is reduced by 60%. A reduction of 60% of the acetate excretion by overexpression of PPC was also obtained for *E. coli* ML308 by Holms [61]. Knocking out *ppc* has a negative effect on the overall cell metabolism: growth rate is impaired and the excretion of undesirable metabolites increases [60, 62, 63].

Noronha et al. [55] showed that the TCA cycle/PPC shunt flux ratio differed between a low acetate producer, *E. coli* BL21 and a high acetate producer, JM109. The PPC shunt is active in BL21 and inactive in JM109. This was confirmed by Yang et al. [57]. In contrast to the wild type, *ppc* overexpression mutants show more activity of the glyoxylate bypass [57] making a higher flux through the Krebs cycle possible. This means that the ratio PPC bypass:Krebs cycle will decrease strongly. According to Yang et al. [57], this ratio is very important for the production of acetate.

It is generally accepted that PPC activity strongly regulates the PCK activity. In the wild type, PPC is more active than PCK, first, to compensate for the activity of PCK and second, to supply the Krebs cycle with sufficient intermediates. Inactivation of *pck* leads immediately to a decrease in PPC activity, since compensation of PCK is no longer needed [57]. Overexpression of *pck* deregulates this system, causing an increase of acetate production.

Phosphotransacetylase (*pta*) and acetate kinase (*ackA*)

Phosphotransacetylase [PTA, EC 2.3.1.8] and acetate kinase [ACKA, EC 2.7.2.1] are the enzymes that

accomplish the production of acetate from acetyl-CoA. Phosphotransacetylase catalyzes the conversion of acetyl-CoA to acetylphosphate with production of CoA-SH. Phosphotransacetylase is activated by pyruvate and inhibited by $NADH+H^+$ [64]

$$\text{acetyl - CoA} + P_i \xrightarrow{\text{PTA}} \text{acetylphosphate} + \text{CoA - SH}$$

Acetate kinase uses the product formed by phosphotransacetylase as substrate; this is the last step of the acetate pathway.

$$\text{acetylphosphate} + \text{ADP} \xrightarrow{\text{ACKA}} \text{acetate} + \text{ATP}$$

Both reactions are reversible. As such, the cell can convert acetate to acetyl-CoA and subsequently use it for biosynthesis reactions.

Mutations in both *pta* and *ackA* have frequently been investigated [2, 11, 54, 65]. All data report a strong reduction of acetate production, when *ackA* and/or *pta* are eliminated. This is at the expense of the growth rate and is accompanied by an increase in the production of other fermentation products such as lactate and formate. Though lactate and formate are less toxic to the cells, they are still disadvantageous for cell growth.

Acetyl-CoA synthetase (*acs*)

When glucose is used in high cell density cultures, acetate is produced and excreted in the medium. Acetyl-CoA synthethase [ACS, EC 6.2.1.1] can use the re-absorbed acetate and convert it to acetyl-CoA via a two-step reaction scheme. These reactions are irreversible and thus they are only responsible for acetate consumption and not for acetate production.

$$\text{acetate} + \text{ATP} + \text{CoA - SH} \xrightarrow{\text{ACS}} \text{acetyl - CoA} + \text{AMP} + \text{PP}_i$$

The enzyme has a strong affinity for acetate (Km of 200 μM), which allows it to function at low acetate concentrations, but it is inhibited by glucose [66, 67]. On the other hand, the reversible *ackA-pta* pathway can assimilate acetate only at high acetate concentrations [15].

Contiero et al. [11] investigated the effect of the deletion of *acs* on the growth on glucose at high cell densities in fed-batch fermentations. No clear conclusion could be drawn from their research. It only indicated that acetyl-CoA plays a key role in accomplishing high cell densities and has no or little importance during normal growth.

The overexpression of *acs* in *E. coli* resulted in a significant reduction in acetate formation during glucose metabolism. It also enhanced the assimilation of acetate when used as the sole carbon source. These characteristics guarantee *acs* overexpression as a positive approach to coping with acetate in *E. coli* fermentations [68].

Pyruvate oxidase (*poxB*)

Pyruvate oxidase [POXB, EC 1.2.2.2] catalyzes the oxidative carboxylation of pyruvate to acetate and CO_2 [69]. This 'non-essential' enzyme is a part of the respiratory chain.

$$\text{pyruvate} + \text{H}_2\text{O} + \text{ubiquinone} - 8 \xrightarrow{\text{POXB}} \text{acetate} + \text{CO}_2 + \text{ubiquinol - 8}$$

An elevated intracellular concentration of pyruvate activates this enzyme, suggesting that POXB regulates the flux partitioning of pyruvate, presumable to reduce the carbon flux toward acetyl-CoA in order to maintain the intracellular pool of CoA for other metabolic functions [10]. Abdel-Hamid et al. [10] investigated the function of *poxB* in *E. coli* by knockout mutants. Inactivation of *poxB* resulted in a decrease of 24% in the carbon converted into biomass. The amount of carbon necessary for energy production increased with 23%. They concluded that pyruvate oxidase is essential for a good overall metabolism functioning. They advised against using *poxB* as target to decrease acetate production. However, Causey et al. [100], have reported the beneficial effect of $poxB^-$ mutation on pyruvate production and cell growth. The relationship between POXB and acetate formation has been studied. Vemuri et al. [70] studied the physiological response of *E. coli* central metabolism to the expression of heterologous pyruvate carboxylase (PYC) in the presence or absence of pyruvate oxidase. The presence of PYC activity in *E. coli* substantially increases the cell yield from glucose, particularly for a *poxB* mutant, biomass, which appears to be derived directly or indirectly from acetate [70]. Recently, a *poxB* knockout strain (and also knockouts in *ldhA* and *pflB* genes) demonstrated significantly reduced acetate formation when the strain was subjected to oscillatory oxygenation [71].

Citrate synthase (*gltA*)

Citrate synthase [CS, EC 2.3.1.1] is the first enzyme of the Krebs cycle. It delivers acetyl-CoA in the cycle via binding with oxaloacetate. During this reaction one molecule of citrate is formed.

$$\text{oxaloacetate} + \text{acetyl - CoA} \xrightarrow{\text{CS}} \text{citrate} + \text{CoA - SH}$$

Citrate synthase is inhibited by α-ketoglutarate and activated by NADH+H$^+$ [56].

This reaction is often indicated as the rate-limiting step of the Krebs cycle. Overexpression of *gltA* showed a decrease of acetate production but not its real elimination [60]. Knocking out *gltA* caused a strong increase in acetate production, accompanied by an increase in formate and pyruvate excretion [72].

Isocitrate lyase (*aceA*), malate synthase (*aceB*) and isocitrate dehydrogenase (*icd*)

Isocitrate lyase [ICL, EC 4.1.3.1] and malate synthase [MS, EC 2.3.3.9] catalyze the reactions of the glyoxylate bypass. Isocitrate lyase converses isocitrate to one molecule succinate and one molecule glyoxylate.

$$\text{isocitrate} \xrightarrow{\text{ICL}} \text{glyoxylate} + \text{succinate}$$

Malate synthase converts glyoxylate and acetyl-CoA into one molecule malate.

$$\text{glyoxylate} + \text{H}_2\text{O} + \text{acetyl - CoA} \xrightarrow{\text{MS}} \text{malate} + \text{CoA - SH}$$

Besides their role in the Krebs cycle, oxaloacetate and α-ketoglutarate are also used for further biosyntheses. This can cause an exhaustion of the Krebs cycle intermediates, because of the continuous need for those essential intermediates. The glyoxylate bypass has as a goal to provide the Krebs cycle with additional oxaloacetate. The switch over from the Krebs cycle to the glyoxylate bypass occurs at the isocitrate dehydrogenase [IDH, EC 1.1.1.42] step.

$$\text{isocitrate} + \text{NADP}^+ \xrightarrow{\text{IDH}} \alpha\text{ - ketoglutarate} + \text{NADPH} + \text{H}^+ + \text{CO}_2$$

Isocitrate dehydrogenase has more affinity for isocitrate than isocitrate lyase. This regulation takes place by reversible phosphorylation of isocitrate dehydrogenase under the influence of the intracellular oxaloacetate level. At high levels of oxaloacetate, isocitrate dehydrogenase is phosphorylated; this phosphorylated form of isocitrate dehydrogenase is not active and as a result, the glyoxylate shunt is activated [57].

Aoshima et al. [73] found that knocking out isocitrate dehydrogenase results in an increase of citrate. El-Mansi et al. [74] tried to delete the glyoxylate shunt by overexpression of isocitrate dehydrogenase. Because of this, the flux through isocitrate lyase decreased, but the intracellular pool of isocitrate became exhausted; they concluded that isocitrate dehydrogenase is not the rate-limiting step in the Krebs cycle. Farmer and Liao [17] stimulated the flux through the glyoxylate shunt by inactivation of the *fadR* operon. This operon negatively controls the expression of isocitrate lyase and malate synthase. Acetate production decreased by 13% from stimulating the glyoxylate shunt.

Yang et al. [57], concluded that the glyoxylate bypass is important in the regulation of the ratio PPC-shunt/Krebs cycle. A high ratio should give a higher acetate production. When the oxaloacetate concentration in the cell is too low, the balance between the glycolysis and the Krebs cycle is deregulated, causing acetate production. It has also been observed that the glyoxylate shunt is active in a low acetate producer, while it is inactive in a high acetate producer [55].

Alterations in the coenzyme pools

Most current metabolic engineering studies have focused on enzyme levels and on the effect of amplification, addition, or deletion of a particular pathway. When enzyme levels are not limiting, the availability and occurrence of coenzymes can become limiting. It is conceivable that in coenzyme-dependent production systems, coenzyme availability and the proportion of coenzyme in the active form may play an important role in dictating the overall process yield. Hence, the manipulation of these coenzyme levels may be crucial in order to further increase production [75–78].

NADH+H$^+$/NAD$^+$

NAD$^+$ plays a significant role in primary metabolism. It is a coenzyme of more than 300 redox reactions. By using this coenzyme, the cell can maintain its redox state in balance. NAD$^+$ and NADH+H$^+$ play a major role in catabolism. To catabolize glucose into precursors, NAD$^+$ is used as coenzyme and converted to NADH+H$^+$. In anabolism, NADP$^+$ and NADPH+H$^+$ occur more frequently, but with a similar function. The cell regenerates the produced NADH+H$^+$ into NAD$^+$ by the reduction of oxygen (under aerobic conditions), or via another oxidizing product or via fermentation. It is also generally known that the ratio NADH+H$^+$/NAD$^+$ regulates the expression of certain genes, such as *adhE*, coding for alcohol dehydrogenase [EC 1.1.1.1], and the activity of certain enzymes, such as the enzymes of the pyruvate dehydrogenase complex [75].

Berrios-Rivera et al. [75] investigated mainly the alterations in the ratio NADH+H$^+$/NAD$^+$. Under aerobic

conditions, formate was added to activate pathways that normally do not function. The results depended on whether the formate dehydrogenase was endogenous(originating from *Candida boidinii*). Adding formate to the strain with the endogenous formate dehydrogenase under aerobic conditions resulted in a large increase in the acetate production compared to the strain with the heterologous enzyme, where a small increase of the acetate production was observed [75].

Acetate overflow at high glucose consumption rates is believed to result from an enzymatic limitation in the TCA cycle causing excess carbon from glycolysis to be shunted to acetate or from a saturation of the respiratory pathways used to reoxidize NADH [46]. Since glycolysis and the TCA cycle generate NADH while acetate formation does not, saturation of NADH oxidation at high glucose consumption rates could cause the cell to form acetate in order to modulate the redox balance [45]. Recently a strong link was demonstrated between redox ratio (in vivo molar concentration ratio NADH/NAD) and acetate overflow metabolism in *E. coli* [45]. The authors revealed that the initiation of acetate overflow metabolism occurred above a critical NADH/NAD ratio of 0.06. In addition, the acetate production could be delayed by the expression of the heterologous NADH oxidase. Expression of the heterologous NADH oxidase coupled with the deletion of the regulatory *arcA* gene in *E. coli*, increased the glycolytic flux and reduced acetate production [46]. The presence of the heterologous NADH oxidase or the absence of ArcA reduced acetate about 50% and increased the recombinant protein production by 10–20%. The presence of the heterologous NADH oxidase in the *arcA* knockout strain eliminated acetate production entirely in batch fermentations and resulted in a 120% increase in the recombinant protein production.

Coenzyme A (CoA-SH)

A second important type of coenzyme is coenzyme A and its derivates (acetyl-CoA, succinyl-CoA,…). Acetyl-CoA is an essential intermediate in many energy-yielding processes. More than 100 different reactions of central metabolism depend on this substrate. It is the main source of activity of the Krebs cycle [78].

The intracellular pool contains mainly short chain CoA-thioesters such as acetyl-CoA and succinyl-CoA. CoA-thioesters of long chain fatty acids form the intermediates of the β-oxidation route and in the production of phospholipids. Besides the major role of CoA-SH in these pathways, it has a substantial regulatory effect. CoA-SH inhibits or activates reactions of the central metabolism and of the fatty acid biosynthesis [79].

The ratio acetyl-CoA/free acetyl-CoA is constant in *E. coli* cells grown on glucose. This ratio might regulate certain enzymes of the central metabolism [56].

San et al. [78] investigated the production and availability of CoA-SH by means of the biosynthesis of isoamylacetate. This pathway does not occur in *E. coli* wild type cells. Acetyl-CoA is used as substrate for the production of isoamylacetate and thus manipulation of the CoA-SH pool influences the production of isoamyl acetate. Pantothenate kinase is the rate-limiting step in the CoA-SH production. This enzyme is inhibited by CoA-SH and acetyl-CoA. San et al. [78] observed small differences in the central metabolism when *coaA* is overexpressed. Acetate and ethanol concentration increased hardly with respect to the isoamyl acetate level, which increased three times.

Alteration in ATP level

Metabolic control theory postulates that flux control could be shared by many enzymes in a pathway and that control could also reside outside the pathway, for instance, in the process that consumes the ATP generated in the glycolysis (ATP demand). Koebmann et al. [80] investigated whether ATP consumption by cellular processes determines the steady-state flux through glycolysis, by increasing the current ATP consumption rate. Therefore, they introduced an ATP-consuming process that does not interfere with other aspects of metabolism. The added ATP activity resulted in up to 70% increase in the rate of glycolysis and they estimate that major control (>75%) resides outside the glycolysis, i.e., in enzymes that consume ATP.

Conclusion and perspectives

Over the last 20 years, substantial research efforts have been expended on reducing acetate accumulation during aerobic growth of *E. coli* on glucose. From the onset it was clear that this quest would not be simple. Simple deletion of the acetate pathway reduced acetate accumulation, but other by-products were formed. From the current state of the literature, we can conclude that reduction of acetate requires a multigene action. In particular, one has to pay attention to the regulation of futile cycles, anapleurotic pathways, coenzyme levels, acetate producing pathways and ATP-consuming pathways. The expression of the heterologous NADH oxidase in an *arcA* knockout strain seems promising. However, the intuitive prediction of the manipulation consequences of several genes is difficult. In most cases the construction of a producer strain did not turn out to be as straightforward as was initially anticipated.

Indeed, in complex metabolic networks, it is often a difficult task to predict ad hoc the impact, both qualitatively and quantitatively, of a genetic intervention [81]. Moreover, as the focus in metabolic engineering is shifting from massive overexpression and inactivation of genes toward the fine-tuning of gene expression [82–90], the need for a reliable, quantitative predictor, i.e., a model, is rapidly growing. The use of metabolic flux analysis is vital here. Metabolic models allow a better prediction of genetic interventions and can help to predict the construction of the ideal *E. coli* phenotype.

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