Communication to the Editor

Enforced ATP futile cycling increases specific productivity and yield of anaerobic lactate production in *Escherichia coli*

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Running title: ATP wasting to improve yield and productivity

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Abstract

The manipulation of cofactor pools such as ATP or NAD(P)H has for long been recognized as key targets for metabolic engineering of microorganisms to improve yields and productivities of biotechnological processes. Several works in the past have shown that enforcing ATP futile cycling may enhance the synthesis of certain products under aerobic conditions. However, case studies demonstrating that ATP wasting may also have beneficial effects for anaerobic production processes are scarce. Taking lactic acid as an economically relevant product, we demonstrate that induction of ATP futile cycling in *Escherichia coli* leads to increased yields and specific production rates under anaerobic conditions, even in the case where lactate is already produced with high yields. Specifically, we constructed a high lactate producer strain KBM10111 (= MG1655 *ΔadhE::Cam ΔackA-pta*) and implemented an IPTG-inducible overexpression of *ppsA* encoding for PEP synthase which, together with pyruvate kinase, gives rise to an ATP consuming cycle. Under induction of *ppsA*, KBM10111 exhibits a 25% higher specific lactate productivity as well as an 8% higher lactate yield. Furthermore, the specific substrate uptake rate was increased by 14%. However, trade-offs between specific and volumetric productivities must be considered when ATP wasting strategies are used to shift substrate conversion from biomass to product synthesis and we discuss potential solutions to design optimal processes.

In summary, enforced ATP futile cycling has great potential to optimize a variety of production processes and our study demonstrates that this holds true also for anaerobic processes.

Key words: cofactor engineering, ATP futile cycling, metabolic engineering, product yield, productivity, lactate

Main Text *(Unstructured as demanded for Communications to the Editor)*

The sustainable production of biofuels and valuable chemicals by microorganisms is a primary goal of metabolic engineering. Yield and productivity are key performance parameters of biotechnological processes that need to be continuously improved to become competitive with conventional chemical synthesis of desired compounds. The manipulation of cofactor pools, such as ATP, NAD(P)H, CoA, or CO_2 has been recognized as one important target for metabolic engineering (Chao and Liao 1994; de Kok et al. 2012; Holm et al. 2010; Lan and Liao 2012; Patnaik et al. 1992; San et al. 2002). In fact, many genetic manipulations seek to directly or indirectly affect the pool of NAD(P)H or/and ATP with the goal to enhance synthesis of a desired product. In the extreme case, product synthesis becomes mandatory for cell growth (growth-coupled product synthesis (Feist et al. 2010)) because, after appropriate interventions, it remains as the only way to balance cofactors. Examples for direct targeting of the NAD(P)H pool in *Escherichia coli* were given by Chemler et al. (2010) demonstrating that increased NADPH availability is beneficial for production of polyphenols, and by Singh et al. (2011) showing the favorable effect of a higher NADH supply for succinate production. Regarding the manipulation of the ATP level, different effects have been observed with respect to productivity and yields. On the one hand, increasing the supply of ATP has been reported to increase, for example, growth rate, succinate synthesis (Singh et al. 2011), or production of recombinant proteins (Kim et al. 2012). On the other hand, studies analyzing the effects of decreased ATP supply through enhanced futile cycling (Chao and Liao 1994; Patnaik et al. 1992) or ATP hydrolysis (Causey et al. 2003; Koebmann et al. 2002) reported increasing production rates and yields of fermentation products or/and elevated glucose consumption under *aerobic* conditions. Surprisingly, whereas usage of ATP wasting strategies has been tested and discussed for

improved synthesis of certain compounds under aerobic conditions, we could not find experimental studies addressing the effect of enforced ATP futile cycling on product synthesis under *anaerobic* conditions, the preferred *modus operandi* for industrial fermentations. Taking lactate as an example for an industrially relevant commodity chemical (Datta and Henry 2006), a simple stoichiometric analysis reveals that synthesis of lactate from glucose by *E. coli* is neutral with respect to redox (NAD(P)H) balance but leads to net ATP production (maximum of 2 mol ATP / mol glucose consumed and 1 mol ATP / mol lactate produced). The motivation to use ATP wasting for lactate synthesis is the following: if lactate is the major fermentation product, some amount of the consumed substrate will be directed to lactate to generate ATP which is required for (i) biomass synthesis (for which the rest of the consumed substrate is used) and for (ii) non-growth-related ATP consumption. Therefore, a higher unspecific ATP consumption, induced, for example, by an ATP wasting strategy, should divert a larger fraction of the substrate to lactate synthesis and thereby improve the performance of a lactate producer strain.

This was also suggested in a recent computational study (Hädicke and Klamt 2010) where we predicted that, besides the well-known knockouts in competing pathways producing alternative products, increased (unspecific) ATP consumption could have a beneficial effect on production of certain fermentation products under anaerobic conditions. A related prediction proposed in this work was overexpression of the *ppsA* gene (encoding the phosphoenolpyruvate (PEP) synthase) to enhance the flux from pyruvate to PEP. Using this target for improving lactate production appears at a fist glance somewhat counter-intuitive since this could potentially lower the amount of pyruvate, the precursor for lactate production. PEP synthase requires ATP as cofactor in which both phosphoanhydride bonds are broken yielding thus AMP:

$$ATP + pyruvate + H_2O \rightarrow AMP + PEP + Pi.$$

AMP can be regenerated to ADP, e.g. through the adenylate kinase under consumption of another ATP:

$$AMP + ATP \rightarrow 2\ ADP.$$

The reverse (i.e., glycolytic) direction from PEP to pyruvate is catalyzed by the pyruvate kinase under production of one ATP

$$PEP + ADP \rightarrow Pyruvate + ATP.$$

This explains the suggestion of *ppsA* as an overexpression target: the three reactions form a cycle along which one ATP is consumed in the net, hence, ATP wasting is effectively enforced as a design principle. Note that this futile cycle was also used by (Patnaik et al. 1992) with the difference that we consider here anaerobic conditions, in contrast to the aerobic experiments investigated in (Patnaik et al. 1992).

With the present study we thus aimed to examine whether ATP wasting via pyruvate kinase / PEP synthase futile cycling holds indeed potential to increase productivity and yields of anaerobic fermentation processes. Focussing on lactate as the relevant product we used the *E. coli* strain KBM10111 (a derviative of MG1655 featuring deletions of Δ*adhE and* Δ*ackA-pta*) where byproduct excretion of ethanol, formate, acetate, and succinate is almost completely blocked whereas lactate is, apart from biomass, the main product under anaerobic conditions. We introduced plasmid *pRR48c* (Parkinson and Houts 1982) carrying the endogenous *ppsA* gene under control of an IPTG inducible promoter into KBM10111 thus enabling overexpression of PEP synthase and thereby implementation of the ATP futile cycle.

Anaerobic growth of KBM10111/pRR48+*ppsA* on glucose in batch experiments revealed that the induction of *ppsA* increases the specific rates of glucose uptake by 14% and of lactate excretion by 25% (Table I). The increase in lactate yield appears to be smaller (8%), however, given that KBM10111/pRR48+*ppsA* without *ppsA* induction already reaches ~80% of the maximum lactate yield, this improvement is still significant. As expected, enhanced lactate production is accompanied by a reduced growth rate (-29%) and reduced biomass yield (-27%). These results indicate that overexpression of *ppsA* establishes indeed a futile cycle via pyruvate kinase and PEP synthase leading to the desired effect that the cell shifts carbon flux from biomass to lactate to account for the increased ATP demand. Apart from this shift, the increased specific uptake rate of glucose (which is in line with the findings of (Patnaik et al. 1992) and (Koebmann et al. 2002) represents another desired effect contributing to the significant increase of the specific lactate production rate. Further studies will be required to clarify the cause of the elevated glucose uptake rates as this could be an effect of lowered ATP levels but also of increased PEP levels (obtained by the action of PEP synthase) which could accelerate glucose uptake via the PTS system.

Other products were found to be not present (including acetate, formate, ethanol) or in very small amounts only (succinate). Using a genome-scale metabolic network model of *E. coli* (Feist et al. 2007) and the measured extracellular rates we estimated the non-growth associated ATP maintenance demand first *without* (~9.7 mmol/(gDW h) which is close to the value of 8.39 mmol/(gDW h) reported by (Feist et al. 2007)) and then *with* PEP synthase overexpression (~18.5 mmol/(gDW h)). The latter value is about twice as high as without induction; this increase can be attributed to the wasted amount of ATP in the established futile cycle. The overall changes in the major metabolic fluxes upon ATP futile cycling are summarized in Figure 1.

Our findings confirm the assumption that ATP futile cycling may increase productivity and yield of lactate production in *E. coli*. However, the reduced growth rate causes inevitably also the effect that less biomass is available to synthesize the desired product. This can be seen in Figure 2: at the beginning, the (volumetric) glucose consumption and lactate production are almost identical for both conditions or even slightly higher with *ppsA* overexpression. Here, the higher specific substrate uptake rate under ATP wasting counter-balances the smaller amount of biomass produced. However, at later time points, the volumetric rates start to become larger if ATP futile cycling is not induced. This effect is a result of the inherent trade-off between high volumetric rates (requiring large amounts of biomass and thus a high growth rate) vs. high product yields and specific rates. Over longer time periods, the volumetric production rates will always be governed by the growth rate of the respective strain.

There are different ways how one can deal with these trade-offs in batch processes. One approach would be to decouple growth and production phase, that is to use a two-stage (dual-phase) process (Anesiadis et al. 2008; Gadkar et al. 2005; Soma et al. 2014) where one lets the cells initially grow as fast as possible (without ATP futile cycling) before, in the second phase, product synthesis is maximized by full induction of ATP futile cycling. If one prefers a single-phase process, tunable promoters (Mijakovic et al. 2005) might be used to adjust the flux through the futile cycle in such a way that a (user-defined) optimal trade-off between product yield and specific production rate on the one hand and biomass yield and volumetric production rate on the other hand is achieved. Only if the cell could counteract the loss of ATP by increasing the substrate uptake by the amount required to synthesize the lost ATP, then ATP futile cycling would effectively increase yield and specific productivity of lactate synthesis to the expense of biomass yield - but *without* loss of volumetric productivity. In our experiments (similar as

observed by (Chao and Liao 1994; Patnaik et al. 1992; Koebmann et al. 2002), we have indeed seen that *E. coli* increases the uptake of glucose under induction of the ATP futile cycle, however, the original growth rate could not be fully restored.

In summary, we could demonstrate that ATP futile cycling may help to increase product yields and specific productivities of production processes with *E. coli* even under anaerobic conditions. Together with the results obtained by others under aerobic conditions (Chao and Liao 1994; Koebmann et al. 2002; Patnaik et al. 1992), we believe that ATP futile cycling holds generally great potential as a tool to optimize bioprocesses for a variety of products and microorganisms. For example, in a theoretical study, Erdrich et al. (2014) discussed recently the use of ATP wasting for improving ethanol synthesis in cyanobacteria. The basic principle of using ATP futile cycles as a metabolic engineering strategy to boost yields and productivities is illustrated in Figure 1. A natural requirement is that synthesis of the desired product from the substrate must lead to net formation of ATP, ideally with high amounts. The ATP balance must be carefully analyzed for the respective process conditions and organisms, since energetic costs for product excretion may vary for different species and process (e.g. pH) conditions (de Kok et al. 2012). Furthermore, under anaerobic conditions, the cofactors NAD(P)H should ideally be balanced with product synthesis since otherwise further products need to be excreted by the cell to achieve a balanced redox state.

Methods

Strains and cultivation

As suitable production host for lactate, we chose the *E. coli* strain KBM10111 (= MG1655 Δ*adhE::cat* Δ*ackA-pta*) where byproduct excretion of ethanol and acetate is already suppressed. Deletions were introduced into MG1655 and the resistance gene of the first knock-out was eliminated as described by (Datsenko and Wanner 2000). Deletions comprise the complete genes leaving only the start codon and the last 6 codons. pRR48+*ppsA* was constructed by PCR-amplifying *ppsA* (encoding for PEP synthase) from MG1655 chromosomal DNA and cloning of the PCR product into plasmid *pRR48c* (Parkinson and Houts 1982) so that *ppsA* is controlled by the IPTG inducible *tac* promoter thus allowing overexpression of *ppsA*. Together with the constitutional activity of the pyruvate kinase (*pykF)* a futile cycle is thus established serving as an artificial ATP sink. The resulting strain KBM10111/pRR48+*ppsA* was cultivated in four biologically independent anaerobic batch experiments with and without induction of *ppsA*.

For growth assays a standard defined medium (SDM) (Tanaka et al. 1967) at pH 7.0 containing 4g/l glucose as sole carbon was used. Ampicillin was added at 10µg/mL. To supply the anaerobic cultures with CO_2 11mM Na_2CO_3 was added to the growth medium prior to inoculation. *ppsA* expression from the *tac* promoter was induced by adding 100µM IPTG to the medium (also prior to inoculation).

For growth assays a single colony from an LB_0 Amp plate was inoculated in 10mL LB_0Amp and incubated at 37°C with shaking for a few hours. Afterwards 10mL of SDM were inoculated with 50µL of the LB_0 culture and incubated overnight at 37°C with slow agitation to avoid sedimentation. Cells from this overnight culture were washed and inoculated to about 10^8

cells/mL in 80mL SDM. Cultures were incubated at 37°C and were slowly shaken to avoid sedimentation. To assure anaerobic growth, flasks were sealed and continuously sparged with N_2. Culture growth was monitored by measurement of optical density at 420nm, with one OD_{420} correlating to $5*10^8$ cells/mL. Culture samples were taken at defined time points with the help of a syringe.

Concentration measurements

Concentrations of glucose and lactate as well as of the other fermentation products in sample supernatants were determined by using the corresponding enzymatic test kits from r-biopharm. For calculating biomass a factor of 0.22g per OD_{420} was applied.

Calculation of rates

The metabolism was assumed to be in a pseudo-steady-state within the first 8h (see Figure 2) and the extracellular rates were calculated from this time span as described elsewhere (Hädicke et al. 2013). The non-growth associated ATP maintenance demand was calculated by metabolic flux analysis using a genome-scale stoichiometric model of *E. coli* (Feist et al. 2007) and the calculated growth rate and excretion rates. We assumed that the cell converted the substrate optimally to produce biomass and ATP. The predicted amount of excess ATP produced by the cells without futile cycling was considered as the non-growth associated ATP demand; in the case with induced futile cycle this number includes in addition the amount of ATP wasted in the futile cycle. All calculations were performed using *CellNetAnalyzer* (Klamt et al. 2007).

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Table I Calculated growth rate (μ), extracellular rates (v_{Glc}, v_{Lac}), non-growth associated ATP consumption (for maintenance processes; $v_{ATPmaint}$) and product yields (Y) in the strains without (-IPTG) and with (+IPTG) *ppsA* induction (see also text and Methods).

	- IPTG	+ IPTG
μ [h^{-1}]	0.196	0.139 (-29.1%)
$v_{Glucose}$ [mmol / (gDW h)]	13.3	15.2 (+14.3%)
$v_{Lactate}$ [mmol / (gDW h)]	20.2	25.2 (+24.8%)
$Y^{Biomass/Glucose}$ [g/g]	0.075	0.055 (-26.7%)
$Y^{Lactat/Glucose}$ [g/g]	0.796	0.863 (8.4%)
$v_{ATPmaint}$ [mmol / (gDW h)]	9.7	18.5 (+8.8 mmol/(gDW h))

Figure legends

Figure 1 Principle of using ATP futile cycling to increase product yield and specific productivities: (a) without and (b) with futile cycling. Elevated ATP wasting through futile cycling is counterbalanced by the cell by shifting flux from biomass to product synthesis to obtain more ATP. As a consequence, the product yield increases whereas the biomass yield decreases. The substrate uptake flux may become enhanced to allow faster synthesis of ATP along the pathway from substrate to product.

Figure 2 Concentration profiles for (a) glucose, (b) lactate and (c) biomass during cultivation of KBM110111/pRR48+*ppsA* with (red) and without (green) induced *ppsA* overexpression. The data points are averaged values from four biologically independent experiments. Error bars represent the empirical standard deviations. *ppsA* production was induced by adding 100µM IPTG to the medium prior to inoculation.