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Distinct transcriptional regulation of the two *Escherichia coli* transhydrogenases PntAB and UdhA

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Transhydrogenases catalyse interconversion of the redox cofactors NADH and NADPH, thereby conveying metabolic flexibility to balance catabolic NADPH formation with anabolic or stress-based consumption of NADPH. *Escherichia coli* is one of the very few microbes that possesses two isoforms: the membrane-bound, proton-translocating transhydrogenase PntAB and the cytosolic, energy-independent transhydrogenase UdhA. Despite their physiological relevance, we have only fragmented information on their regulation and the signals coordinating their counteracting activities. Here we investigated PntAB and UdhA regulation by studying transcriptional responses to environmental and genetic perturbations. By testing *pntAB* and *udhA* GFP reporter constructs in the background of WT *E. coli* and 62 transcription factor mutants during growth on different carbon sources, we show distinct transcriptional regulation of the two transhydrogenase promoters. Surprisingly, transhydrogenase regulation was independent of the actual catabolic overproduction or underproduction of NADPH but responded to nutrient levels and growth rate in a fashion that matches the cellular need for the redox cofactors NADPH and/or NADH. Specifically, the identified transcription factors Lrp, ArgP and Crp link transhydrogenase expression to particular amino acids and intracellular concentrations of cAMP. The overall identified set of regulators establishes a primarily biosynthetic role for PntAB and link UdhA to respiration.

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INTRODUCTION

To fuel the about 300 anabolic reactions during growth of *Escherichia coli*, central carbon metabolism supplies building blocks, energy and redox power at appropriate rates and stoichiometries (Michal, 1999). The transfer of electrons between catabolism, respiration and anabolism as well as removal of reactive oxygen species is achieved by the redox cofactors NADH and NADPH (Ritz & Beckwith, 2001), which are among the most highly connected metabolites (Holm *et al.*, 2010). Both cofactors have distinct physiological roles. In the presence of an external electron acceptor, the primary role of NADH is in respiratory ATP generation

via oxidative phosphorylation, whereas it is primarily a catabolic byproduct during anaerobic fermentation that must be reoxidized through formation of reduced fermentation end products such as lactate or ethanol. The chemically similar redox cofactor NADPH, in contrast, mainly drives anabolic reductions (Fuhrer & Sauer, 2009). To fulfil these distinct functions, cells maintain the NADPH-to-NADP⁺ ratio in a more reduced state than the NADH-to-NAD⁺ ratio (Harold, 1986).

For most investigated microbes, the major sources of NADPH during hexose metabolism are the oxidative pentose phosphate (PP) pathway and the isocitrate dehydrogenase reaction in the tricarboxylic acid (TCA) cycle (4). In some organisms, such as *Saccharomyces cerevisiae*, the catabolic fluxes through the NADPH-generating reactions match precisely the anabolic requirements for NADPH (Bakker *et al.*, 2001; Blank *et al.*, 2005). Many bacteria, however, encounter conditions where

Abbreviations: PP, pentose phosphate; IHF, integration host factor.

Four supplementary figures and four supplementary tables are available with the online Supplementary Material.

catabolic NADPH formation exceeds their anabolic demand or conditions under which formation will be insufficient (Sauer *et al.*, 2004; Fuhrer *et al.*, 2005), which require additional NADPH-balancing mechanisms. Generally, NADPH-balancing mechanisms can be divided into those that prevent imbalance and others that correct for the imbalance by decoupling NADPH formation from catabolism. Biochemical mechanisms that avoid imbalance include altered catabolic pathway usage, for example, phosphoenolpyruvate–glyoxylate cycle versus TCA cycle usage (Fischer & Sauer, 2003; Haverkorn van Rijsewijk *et al.*, 2011), and altered usage of isoenzymes with distinct cofactor specificities (Doan *et al.*, 2003; Driscoll & Finan, 1997; Zamboni *et al.*, 2004). Decoupling catabolic NADPH formation can be achieved by NAD(H) kinases (Kawai *et al.*, 2001; Outten & Culotta, 2003; Singh *et al.*, 2008), by biochemical redox cycles (Overkamp *et al.*, 2002; Verho *et al.*, 2002) and through transhydrogenases that can transfer electrons directly from NADH to NADP⁺ and vice versa (Sauer *et al.*, 2004).

E. coli is one of very few microbes that possesses two transhydrogenases: the membrane-bound, proton-translocating transhydrogenase PntAB (David *et al.*, 1986) and the cytosolic, energy-independent transhydrogenase UdhA (Boonstra *et al.*, 1999). Both isoforms have been shown to exert distinct functions in redox balancing of *E. coli*. PntAB is a major contributor to NADPH formation during growth on glucose, and mutant data suggest UdhA as an important contributor to NADPH oxidation on substrates such as acetate, whose catabolism produces more NADPH than is required for cell growth (Sauer *et al.*, 2004). Despite their physiological relevance and genetic evidence that both transhydrogenases are not constitutively expressed in *E. coli* (Edgar *et al.*, 2002; Faith *et al.*, 2008; Sherlock *et al.*, 2001; Liu *et al.*, 2005), transcriptional regulation of transhydrogenases and the signals that trigger their expression are poorly understood. Based on reported *E. coli* mRNA expression, metabolite levels and ¹³C-based flux data under eight nutritional conditions (Gerosa *et al.*, 2015), we first demonstrate that transhydrogenase transcription does not correlate with the cellular NADPH demand but suggest rather a growth-related biosynthetic function for PntAB. Using GFP reporter plasmids in 62 transcription factor knockouts, we then systematically identify factors that regulate transhydrogenase expression and demonstrate the availability of extracellular amino acids and growth rate, which, in turn, both affect redox metabolism, as the primary regulation factors. By identifying the transcriptional regulators that regulate both transhydrogenase isoforms, we provide insights into the distinct physiological functions of the two redox-balancing transhydrogenase isoforms, linking PntAB to biosynthesis and UdhA to respiration.

METHODS

Strain and growth conditions. All experiments were performed with *E. coli* BW25113 and its otherwise isogenic mutants from the Keio knockout library (Baba *et al.*, 2006). For clarity, the mutant nomenclature used here reflects the deleted genes. Online biomass and GFP

measurements were conducted in 200 µl 96-well plates in a plate reader (Tecan Infinite Pro 200). Frozen glycerol stocks were used to inoculate Luria–Bertani (LB) complex medium. For cultivation of strains containing GFP reporter plasmids, LB medium of the precultures was supplemented with 50 mg l⁻¹ kanamycin or 25 mg l⁻¹ chloramphenicol depending on the antibiotic resistance cassette present in the strain or the plasmid. All further cultivations were performed without antibiotics. After 6 h of incubation at 37 °C and constant shaking, LB cultures were used to inoculate M9 minimal medium precultures upon overnight cultivation. On the following day, the M9 precultures were used to inoculate 1 : 100 (v/v) for the final experiments.

The M9 medium contained, per litre of deionized water, the following: 0.8 g (NH₄)₂SO₄, 0.5 g NaCl, 7.5 g Na₂HPO₄·2H₂O and 3.0 g KH₂PO₄. The following components were sterilized separately and then added (per litre of final medium): 1 ml of 1 M MgSO₄, 1 ml of 0.1 M CaCl₂, 0.6 ml of 0.1 M FeCl₃·6H₂O, 2 ml of 1 mM filter-sterilized thiamine HCl and 10 ml of a trace element solution containing (per litre) 0.18 g ZnSO₄·7H₂O, 0.12 g CuCl₂·2H₂O, 0.12 g MnSO₄·H₂O and 0.18 g CoCl₂·6H₂O. Filter-sterilized carbon source was added to a final concentration of 5 g per litre. For experiments supplemented with all 20 amino acids, final concentrations in the medium representing the biomass composition are as follows (Zaslaver *et al.*, 2006): alanine, 0.47 mM; arginine, 0.6 mM; asparagine, 0.32 mM; aspartic acid, 0.3 mM; cysteine, 0.3 mM; glutamate, 5 mM; glutamine, 5 mM; glycine, 0.13 mM; histidine, 0.1 mM; isoleucine, 0.3 mM; leucine, 0.3 mM; lysine, 0.3 mM; methionine, 0.3 mM; phenylalanine, 0.3 mM; proline, 2 mM; serine, 4 mM; threonine, 0.3 mM; tryptophan, 0.1 mM; tyrosine, 0.1 mM; valine, 0.3 mM. For experiments with single amino acid additions, amino acids were added to a final concentration of 5 mM. For oxidative stress experiments, the medium was supplemented with paraquat to obtain a final concentration of 10 µM.

Reporter plasmid construction and transformation. The vectors used for this study were low-copy plasmids containing transcriptional fusions. More specifically, the plasmids contain the regulatory region of a specific gene fused to a GFP reporter gene *gfpmut2* as previously described (Zaslaver *et al.*, 2004, 2006). GFP_{MUT2} becomes fluorescent within less than 5 min of transcription initiation and is highly stable and non-toxic in *E. coli* (Zaslaver *et al.*, 2004, 2006). As a regulatory region, the entire intergenic region was taken between two ORFs extending it by 50–100 bp into each of the two flanking ORFs. For the *pntAB* and *udhA* promoter activities, plasmids were used from the Zaslaver library (Zaslaver *et al.*, 2006). As both *pntA* and *pntB* are encoded by the promoter upstream of *pntA*, for this study, the intergenic region upstream of the *pntA* promoter is used and is termed *pntAB*. Chloramphenicol-resistant plasmids for *pntAB* and *udhA* were synthesized by removing the kanamycin cassette from the original plasmids from the Zaslaver library and replacing it with the chloramphenicol resistance marker encoding the chloramphenicol acetyl transferase (Alton & Vapnek, 1979). Replacing the kanamycin with the chloramphenicol cassette had no effect on GFP expression or growth rate for strains carrying the *pntAB* or *udhA* reporter plasmid (data not shown). Two synthetic constitutive promoters, in which all transcription-factor-binding sites had been scrambled in order to quantify the growth-rate-dependent effect of the cellular physiology on promoter activity and a synthetic reporter promoter that is only regulated (activated) by Crp, were obtained from previous studies (Gerosa *et al.*, 2013, 2015).

Online GFP and biomass measurements and promoter activity calculations. Online measurements for all batch experiments in 96-well plates were conducted in a Tecan 96-well plate reader (Tecan Infinite Pro 200) with linear shaking and temperature kept at 37 °C. OD₆₀₀ and GFP (500 nm excitation and 530 nm emission) were measured online every 10 min for each well to follow biomass formation and GFP expression, respectively (Samorski *et al.*, 2005). Background

optical density and fluorescence signal due to the growth medium were subtracted for the Tecan 96-well plate reader. For GFP measurements, additional background fluorescence due to biomass was also subtracted.

Promoter activities and relative mRNA expression levels were calculated using online GFP and optical density measurements. Promoter activities (PA) were calculated during mid-exponential phase (OD_{600} 0.7–1.5) by calculating the optical density-normalized GFP production rate over time:

$$PA = dGFP/(dt \cdot OD) \text{ (Zaslaver et al., 2006)}$$

NADPH-balancing calculations. Previously, ^{13}C -based intracellular fluxes (Gerosa et al., 2015) were used as a basis for all the calculations. The catabolic underproduction or overproduction of NADPH was calculated as the total NADPH formation rate minus the total NADPH consumption rate. The rates of total NADPH formation were determined from the reported net fluxes through the NADPH-generating reactions in the TCA cycle (i.e. isocitrate dehydrogenase) and PP pathway (i.e. glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) and those through the malic enzymes. In the case of the malic enzymes, we assumed either 50% of flux through the NADPH-producing malic enzyme B and 50% through the NADH-producing malic enzyme SfcA or 100% of malic enzyme flux through either isoform. The rate of NADPH consumption was calculated from the NADPH requirements for biomass production (Neidhardt & Bott, 1990) and the growth rate. Error analysis on NADPH production–NADPH consumption was conducted via SE propagation as:

$$NB^{\text{error}} = \sqrt{\left(v_{zwf}^{\text{error}} + v_{gnd}^{\text{error}} + v_{maeB}^{\text{error}}\right)^2 + (NC^{\text{error}})^2}$$

where NB^{error} is the error in catabolic NADPH underproduction or overproduction, v represents the errors in fluxes through the specific NADPH-producing enzymes and NC^{error} is the calculated error in NADPH consumption.

RESULTS

Cellular NADPH demand does not regulate transcription of either transhydrogenase isoform

Previously determined expression data (Gerosa et al., 2015) for *E. coli* WT growth on eight different carbon sources (i.e. galactose, acetate, pyruvate, succinate, glycerol, gluconate, glucose and fructose) showed distinct expression levels for both transhydrogenase isoforms (Fig. 1a). Although *pntAB* promoter activity was highest on glucose, *udhA* promoter activity was low on glucose and highest on succinate and pyruvate. Given that the transhydrogenases are important for balancing redox metabolism in *E. coli* (Sauer et al., 2004; Fuhrer et al., 2005), we hypothesized that the expression of the transhydrogenase-encoding genes *pntAB* and *udhA* might respond to the cellular redox cofactor requirements.

To test this hypothesis, we used ^{13}C -based intracellular flux data reported in Gerosa et al. (2015) to calculate NADPH production through the key NADPH-generating pathways. We then quantified apparent catabolic overproduction or underproduction of NADPH by subtracting the stoichiometric NADPH requirements for biomass formation from the ^{13}C -based NADPH production. When plotting the reported mRNA levels of both *pntAB* and *udhA* (Gerosa et al., 2015) against the magnitude of catabolic NADPH overproduction or underproduction (Fig. 1b), we found no significant correlation of mRNA abundance with the estimated underproduction and overproduction rates. To confirm that the NADPH demand does indeed not regulate transhydrogenase transcription, we grew the two GFP reporter strains on glucose supplemented with 10 μ M paraquat. Continuous generation of reactive oxygen species by

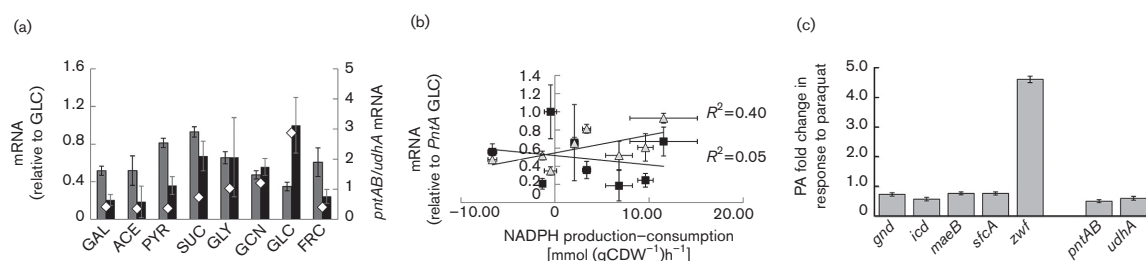


Fig 1. The expression of *pntAB* (black bars) and *udhA* (grey bars) normalized to the WT *pntAB* expression on glucose and ratio between *pntAB* and *udhA* expression (white diamonds) (a), *pntAB* (black squares) and *udhA* (grey triangles) mRNA expression levels plotted against NADPH production–consumption for the WT for growth on eight different carbon sources (b) and promoter activity (PA) fold change for the key NADPH-producing enzymes and *pntAB* and *udhA* during mid-exponential growth on glucose in response to 10 μ M paraquat (c). *pntAB* mRNA expression is the mean of *pntA* and *pntB* mRNA expression normalized to glucose. *pntA* and *pntB* showed identical expression levels (see Fig. S1, available in the online Supplementary Material). For NADPH production–consumption calculations, 50% of malic enzyme flux was assumed via the NADPH-producing malic enzyme B and 50% through isoenzyme SfcA. Correlations assuming full flux through either isoform were also determined (see Fig. S2). GAL, galactose; ACE, acetate; PYR, pyruvate; SUC, succinate; GLY, glycerol; GCN, gluconate; GLC, glucose; FRC, fructose. Values represent the mean of three (a and b) or two (c) biological replicates. Error bars for mRNA expression levels represent the calculated SD of three biological replicates. Error bars for the NADPH imbalance represent the error propagation in the NADPH producing and consuming fluxes.

paraquat increases the cellular NADPH demand for detoxification. Expectedly, we found increased promoter activity of *zwf*, the first gene of the NADPH-producing PP pathway, but no increase in either transhydrogenase-encoding gene (Fig. 1c). The oxidative stress experiment thus provided further evidence that the NADPH demand does not regulate transhydrogenase transcription.

Correlation analysis indicates biosynthetic function for PntAB

If not the catabolic overproduction or underproduction of NADPH, we wondered which other metabolic processes would trigger the distinct transcription of both transhydrogenase isoforms? To identify potential metabolic signals, we correlated transhydrogenase mRNA levels to the previously determined (Gerosa *et al.*, 2015) steady-state concentrations of 41 metabolites, 23 intracellular fluxes and growth rate on the eight carbon sources (Table S1). Generally, *udhA* mRNA levels showed no significant correlation with intracellular metabolites (Pearson correlations <0.80 , $P < 0.05$), whereas *pntAB* mRNA was correlated to phenylalanine (Pearson correlation 0.91). However, mRNA levels of neither isoform correlated with changes in redox cofactor levels or their ratios, indicating that the redox state per se does not regulate transhydrogenase expression.

At the level of flux, *pntAB* mRNA levels correlated slightly positively with glycolysis, whereas *udhA* mRNA levels correlated negatively with fluxes through glycolysis and PP pathway and positively with the late TCA cycle. The only statistically significant correlation, however, was between *pntAB* mRNA and growth rate (Pearson correlation = 0.84, $P = 0.02$), with *pntAB* mRNA levels increasing with increasing growth rates, the major determinant of cellular NADPH consumption (Smith, 1992) (Fig. 2a). Moreover, *pntAB* mRNA levels also correlated with the growth-rate-dependent α -ketoglutarate-to-glutamine ratio (Fig. 2b), which regulates the split between carbon flux into the TCA

cycle versus the NADPH-consuming amino acid biosynthesis (Hart *et al.*, 2011). These results indicate a biosynthetic role for the PntAB transhydrogenase isoform that would be consistent with the previously demonstrated physiological role in supplying the cell with NADPH under conditions of NADPH shortage (Sauer *et al.*, 2004).

Inferring the underlying regulators for *udhA* and *pntAB* transcription

To identify regulators of transhydrogenase expression, we transformed 62 transcription factor deletion mutants (Baba *et al.*, 2006), including all known regulators of the main redox pathways and central metabolism (Table S2), with the *pntAB* and *udhA* GFP reporter plasmids. This library was then grown in microtitre batch cultures on the substrates glycerol, succinate and glucose (Table S3), which that were previously shown to strongly affect *pntAB* and *udhA* expression levels (Fig. 1a). All mutants were able to grow, with the exception of the Crp mutant on succinate and glycerol. Consistent with the hypothesized role in biosynthetic NADPH supply, only the *pntAB* but not the *udhA* promoter activities increased with mutant growth rate (Fig. S3). To identify specific regulators, we next looked for promoter activity outliers in the mutant set for each substrate, following the logic that the deleted transcription factor of such outliers must have been involved in maintaining the appropriate mRNA levels (Fig. 3). Outliers were defined as transcription factor mutants with at least a 50% increase or decrease in promoter activity compared to the WT, in order to minimize transcription factor identification being subjected to potential indirect effects on promoter activities, i.e. growth rate. For *pntAB*, we thereby identified activating roles of the integration host factor (IHF) on all three substrates, the leucine-responsive regulatory protein Lrp on glycerol and glucose, the ferric uptake regulator Fur on succinate and the arginine protein ArgP on glucose. For *udhA*, we identified a repressive role for Lrp on glycerol and glucose and for the anoxic redox control A factor ArcA on glucose. ArcA has recently been shown to activate *pntAB* and repress *udhA* in a redox-dependent fashion (Federowicz *et al.*, 2014). Our findings support *udhA* repression by ArcA; however, we found no ArcA-dependent activation of *pntAB* under our conditions.

Overall, the identified set of transcriptional regulators partially explains the previously described correlation between *pntAB* mRNA levels and growth rate (Fig. 2b). Furthermore, they link UdhA to respiration. The *pntAB*-activating transcription factors Lrp and ArgP are known activators of mainly biosynthetic genes (Calvo & Matthews, 1994; Cui *et al.*, 1996; Bouvier *et al.*, 2008; Ruiz *et al.*, 2011). The *udhA* repressing transcription factor ArcA is a known regulator of respiratory and TCA cycle enzymes (Perrenoud & Sauer, 2005; Iuchi & Lin, 1988) and has previously been shown to actively control respiratory TCA cycle fluxes during aerobic batch growth on glucose (Perrenoud & Sauer, 2005; Haverkorn van Rijsewijk *et al.*, 2011).

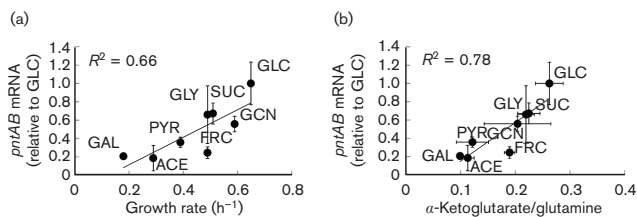


Fig 2. Correlation of *pntAB* mRNA levels with growth rate (a) and the α -ketoglutarate-to-glutamine ratio (b). *pntAB* mRNA expression is the mean of *pntA* and *pntB* mRNA expression normalized to glucose. *pntA* and *pntB* showed identical expression levels (see Fig. S1). GAL, galactose; ACE, acetate; PYR, pyruvate; GLY, glycerol; FRC, fructose; SUC, succinate; GCN, gluconate; GLC, glucose. Error bars for growth rate and mRNA levels represent the calculated SD from three biological replicates.

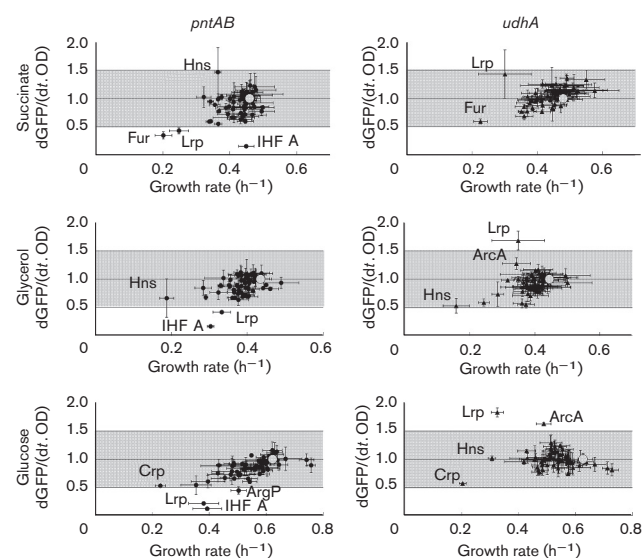


Fig 3. Promoter activities of *pntAB* and *udhA* in *E. coli* WT and 62 transcription factor mutants during growth on three carbon sources. The WT is represented by large circles. Activities were normalized to the WT promoter activities and values represent the mean of two biological replicates; error bars represent the SD. For selected transcription factors, a third biological replicate was conducted and error bars represent the calculated SD of the three biological replicates (Table S3). The grey area indicates a 50% increased or decreased promoter activity relative to the WT.

Inferred regulators link transcription of *pntAB* and *udhA* to signals that sense environmental changes affecting the need for redox cofactors

Since the above identified transhydrogenase regulators Lrp and ArgP are allosterically modulated by leucine and lysine, respectively, amino acids are a likely regulatory trigger. To determine the effect of extracellular amino acids on transhydrogenase transcription, we grew the transcription factor deletion library on glucose supplemented with all 20 amino acids (Fig. 4, Table S4). While promoter activity of *udhA* increased, promoter activity of *pntAB* decreased upon amino acid supplementation, presumably via reduced Lrp activity (Bouvier *et al.*, 2008; Cui *et al.*, 1996). To identify the specific modulators of transhydrogenase transcription, we grew WT *E. coli* containing the *pntAB* or *udhA* GFP reporter plasmid in 20 media supplemented with only one natural amino acid each (Fig. 4). Expectedly, *E. coli* did not grow in the presence of valine as previously described (Leavitt & Umbarger, 1962). Transhydrogenase promoter activities were affected by histidine, methionine, lysine and leucine. Addition of leucine reduced *pntAB* and increased *udhA* promoter activities, presumably via decreased Lrp activity. Addition of lysine also reduced *pntAB* transcription, potentially because of reduced ArgP activity. Methionine activated *udhA* transcription, whereas histidine repressed *pntAB* transcription. Although methionine and

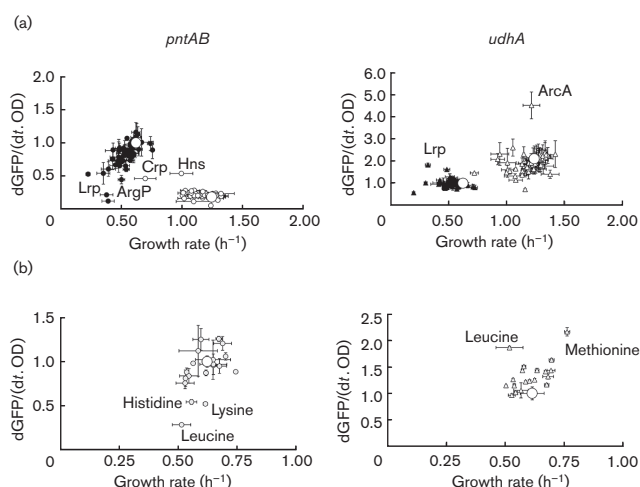


Fig 4. (a) Promoter activities of *pntAB* and *udhA* in 62 transcription factor mutants on glucose supplemented with (white) and without (black) the addition of 20 amino acids. (b) Effect of single amino acid addition on WT *pntAB* and *udhA* promoter activity relative to the WT promoter activity on glucose. The WT promoter activities are depicted as a large white circles. Error bars represent the calculated SD from two biological replicates.

histidine are described to affect Lrp activity (Hart & Blumenthal, 2011), the mechanism is not clear because one would then also expect lower *pntAB* and higher *udhA* expression, respectively, which was not observed.

While we do not have a hypothesis on what caused the strong promoter activity effects of ArcA on *udhA* and Hns and Crp on *pntAB* in the presence of amino acids (Fig. 4), we noted repression of *pntAB* via Crp which was consistent with previous results (Chou *et al.*, 2015). Since Crp activity depends on allosteric binding of intracellular cAMP, which, in turn, responds to growth rate and carbon source availability (Bettenbrock *et al.*, 2007; Haverkorn van Rijsewijk *et al.*, 2011), we wondered whether a growth-rate-dependent cAMP-Crp repression could explain the identified correlation between growth rate and *pntAB* promoter activity (Figs 2 and S3). To establish a direct link from cAMP to *pntAB* transcription via Crp, we determined the effect of extracellular cAMP addition on Crp activity, using a Crp activity reporter plasmid, and on *pntAB* and *udhA* promoter activity (Fig. 5). To distinguish between the indirect effect of the reduced growth rate upon cAMP addition and direct cAMP regulation of promoter activity, we normalized promoter activities by the mean promoter activity of two constitutive plasmids (Fig. S4). Upon cAMP addition, Crp activity increased over twofold. As to transhydrogenase promoter activities, *udhA* promoter activity was only marginally reduced. However, *pntAB* transcription was significantly repressed with increased cAMP additions resulting in approximately 50% reduced promoter activity. Consistent with the previously described physiological

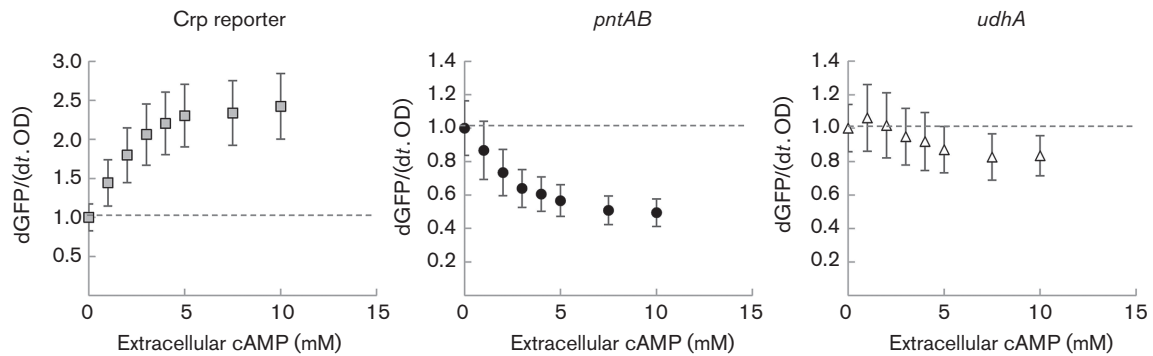


Fig 5. Crp reporter and *pntAB* and *udhA* promoter activity in response to extracellular cAMP. The promoter activities were normalized by the mean promoter activity of two constitutive plasmids (Fig. S4) in order to distinguish between the indirect effect of reduced growth rates upon cAMP addition and the direct effect of cAMP on promoter activities. Promoter activities are relative to the promoter activity without extracellular cAMP, represented by the dashed line. Error bars represent the SD of four biological replicates.

function of PntAB in supplying *E. coli* with NADPH (Sauer *et al.*, 2004), this coupling of *pntAB* transcription to the growth-rate-dependent cAMP-Crp activity (Haverkorn van Rijsewijk *et al.*, 2011; Bettenbrock *et al.*, 2007) potentially facilitates increasing NADPH production with increasing requirements for biosynthesis.

DISCUSSION

Using GFP reporter plasmids, we demonstrated distinct transcriptional regulation of the transhydrogenase isoforms in *E. coli*. Since this regulation was not a function of the balance between catabolic NADPH production and anabolic NADPH consumption, we looked for other cellular traits as potential regulatory triggers. We confirmed the previously suggested function of the membrane-bound PntAB in anabolic NADPH formation through the strong correlation between *pntAB* mRNA levels and growth rate, the main determinant for anabolic NADPH consumption (Smith, 1992), and the α -ketoglutarate-to-glutamine ratio, which determines the flux split between respiratory TCA cycle flux and NADPH-consuming amino acid biosynthesis (Hart *et al.*, 2011). From a screen with 62 transcription factor mutants, we identified Lrp activation and repression of *pntAB* and *udhA* transcription, respectively, which is consistent with microarray data (Hung *et al.*, 2002). Moreover, two distinct groups of transcription factors regulated *pntAB* and *udhA* transcription in a condition-dependent manner. In contrast to *udhA*, whose transcription was only affected (negatively) by the respiratory regulator ArcA, which is consistent with previously reported results (Federowicz *et al.*, 2014), *pntAB* was repressed by HNS and Crp and activated by IHF, ArgP and Fur. At least for the transcription factor Lrp, direct binding to the *pntAB* promoter region has been shown by ChIP-chip experiments (Cho *et al.*, 2008), partially supporting the results presented here. For the

remaining identified transcription factors, direct binding to the promoter regions remains to be demonstrated.

The transhydrogenase-regulating transcription factors appear to respond to at least two signals. The first signal is the availability of amino acids sensed via the transcription factors Lrp and ArgP. ArgP activates *pntAB* transcription, whereas Lrp inversely regulates transcription of both transhydrogenases, activating *pntAB* and repressing *udhA* transcription. The second signal is the intracellular level of cAMP, which is low at high growth rates (Bettenbrock *et al.*, 2007; Haverkorn van Rijsewijk *et al.*, 2011). cAMP is sensed via allosteric binding to Crp and subsequently represses *pntAB* transcription at low growth rates when less NADPH is required for biosynthesis. Notably, both signals represent changes that require redox rebalancing. Increased uptake of amino acids decreases NADPH requirements for cell growth and results in decreased expression of *pntAB* and increased expression of *udhA*. Rapidly growing cells require high NADPH-generating fluxes and the low intracellular levels of cAMP at high growth rates result in reduced Crp repression of *pntAB*. Hence, although the transcriptional regulators of both transhydrogenases do not directly respond to the imbalance between NADPH consumption and NADPH production, they do adjust transcription of PntAB and UdhA in response to specific intracellular and extracellular cues that indicate specific redox cofactor demands.

Since redox homeostasis must be achieved within seconds and transcriptional responses operate on a timescale of minutes, one would expect an additional, more rapid regulation process to control transhydrogenase activity in *E. coli*. For instance, the transcriptional response to oxidative stress in yeast (Chechik *et al.*, 2008) was shown to be preceded by a fast metabolic switch function of glyceraldehyde-3-phosphate dehydrogenase (Ralser *et al.*, 2007, 2009). Within 30 s, the enzyme is inactivated, resulting in blocked

glycolysis and a flux rerouting through the PP pathway. Since we found expression of the soluble transhydrogenase under conditions of NADPH underproduction such as growth on glucose, a condition where deletion of *udhA* has no phenotype (Sauer *et al.*, 2004), it is likely that at least the soluble transhydrogenase UdhA is subject to further, possibly allosteric regulation.

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B. R. B. H. v. R. conceived and designed the study, performed experiments and wrote the manuscript. K. K. performed the cAMP addition and Crp activity experiment. M. H. assisted in the design and supervision of the study. U. S. supervised the study and wrote the manuscript. All authors read and approved the final manuscript.

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