Towards *in vivo* regulon kinetics: PurR activation by 5-phosphoribosyl-α-1-pyrophosphate during purine depletion in *Lactococcus lactis*

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Short-term adaptation to changing environments relies on regulatory elements translating shifting metabolite concentrations into a specifically optimized transcriptome. So far the focus of analyses has been divided between regulatory elements identified *in vivo* and kinetic studies of small molecules interacting with the regulatory elements *in vitro*. Here we describe how *in vivo* regulon kinetics can describe a regulon through the effects of the metabolite controlling it, exemplified by temporal purine exhaustion in *Lactococcus lactis*. We deduced a causal relation between the pathway precursor 5-phosphoribosyl-α-1-pyrophosphate (PRPP) and individual mRNA levels, whereby unambiguous and homogeneous relations could be obtained for PurR regulated genes, thus linking a specific regulon to a specific metabolite. As PurR activates gene expression upon binding of PRPP, the pur mRNA curves reflect the *in vivo* kinetics of PurR PRPP binding and activation. The method singled out the *xpt-pbuX* operon as kinetically distinct, which was found to be caused by a guanine riboswitch whose regulation was overlaying the PurR regulation. Importantly, genes could be clustered according to regulatory mechanism and long-term consequences could be distinguished from transient changes – many of which would not be seen in a long-term adaptation to a new environment. The strategy outlined here can be adapted to analyse the individual effects of members from larger metabolomes in virtually any organism, for elucidating regulatory networks *in vivo*.

**INTRODUCTION**

*Lactococcus lactis*, like many other organisms, has the ability to obtain purine nucleotides in two different ways: either exogenous purine nucleosides and nucleobases are salvaged from the environment by uptake and interconversion, or they are produced intracellularly by the universal biosynthetic pathway utilizing amino acids and ribose (Kilstrup et al., 2005; Martinussen & Hammer, 1995; Martinussen et al., 2010). Previously, we have reported that the presence of exogenous purines simultaneously boosts the growth rate of *L. lactis* by 10 % and lowers purC expression, as detected both at the transcriptional (Jendresen et al., 2012) and the proteomic level (Beyer et al., 2003). Besides increasing the growth rate, the supplementation of purines has been found to increase the intracellular purine nucleotide pool, to decrease the pyrimidine nucleotide pool and to drastically lower the 5-phosphoribosyl-α-1-pyrophosphate (PRPP) pool (Jendresen et al., 2011; Kilstrup et al., 2005; Martinussen et al., 2003). Regulation of purine biosynthesis genes in lactic acid bacteria is dependent on the PurR activator (Kilstrup & Martinussen, 1998; Kilstrup et al., 1998). Genes activated by PurR have been reported to contain a binding motif, which consists of a PurBox-like sequence at a fixed distance from a −10 element (Jendresen et al., 2012; Kilstrup et al., 1998). PurR dependent regulation often involves more complex types of PurBox structures, such as the double-PurBox motif and PurR activated promoters that are not devoid of promoter −35 elements (Jendresen et al., 2012). The activation of PurR is dependent on the presence of the effector molecule PRPP, which has been shown to be closely correlated with the intracellular purine nucleotide level (Jendresen et al., 2011).
Diauxic growth of *Escherichia coli* has been the major paradigm of gene regulation during metabolic shifts, since its description by Jacques Monod in 1941 (Monod, 1941). The most common example of diauxic growth is the exhaustion of glucose from the medium, where the bacterium stalls while its transcriptome is changed to accommodate growth on a new carbon and energy source. Here, we have examined the temporal transcriptome rearrangements during a shift-situation where the bacterium *L. lactis* has exhausted its environment for an exogenous purine source. The temporal changes in intracellular nucleotide pools and concomitant global gene expression levels upon purine depletion during transition from purine salvage to biosynthesis have never been investigated in any bacterium, although such shifts would be likely to occur in natural environments. Transcripts could be grouped into six responsive clusters, and it was found that mRNA levels in two clusters correlated unambiguously with the PRPP levels. One cluster contained PurR regulon members, while the genes in the other cluster belonged to the PyrR regulon involved in pyrimidine biosynthesis.

**METHODS**

**Strains, media and sampling conditions.** *L. lactis* strains are generally grown in M17 media (Terzaghi & Sandine, 1975) supplemented with 1% glucose, but for experimentation, we used the chemically defined media SA (Jensen & Hammer, 1993) supplemented with 1% glucose (GSA). We used the strains MG1363 (Gasson, 1983) and its derivative CBJ53 (attB::P<sup>purC</sup>-lacLM, erm<sup>β</sup>) (Jendresen et al., 2012) expressing β-galactosidase from a purC promoter integrated in the chromosome. The cells from the two strains were grown as balanced cultures in GSA with an excess of 180 µg hypoxanthine (Hx) ml<sup>−1</sup> for 18 generations in a 30 °C water bath. They were then diluted 1000-fold into GSA containing 7.5 Hx ml<sup>−1</sup>, resulting in a final concentration of 7.68 µg Hx ml<sup>−1</sup>, assuming that only a minuscule part of the Hx had been used. Samples for measurement of β-galactosidase were withdrawn from the CBJ53 culture as previously described (Jendresen et al., 2012). At an OD<sub>600</sub> of 0.1, 1.2–2 ml of MG1363 was transferred to 6–10 µl of <sup>32</sup>P<sub>PO<sub>4</sub></sub> in an Eppendorf tube in the same water bath, for quantification of nucleotide and PRPP pools. Measurements of intracellular pools of nucleotides and PRPP were performed as previously described (Jendresen et al., 2011). One hundred microlitres of labelled cells were transferred to Eppendorf tubes and quenched with 20 µl 10 M formic acid precooled to ~20 °C, vigorously mixed, and flash-frozen on dry ice. The samples were subjected to three freeze–thaw cycles between ice-water and dry ice. A media sample was taken and, after centrifugation, a raw (R) sample was withdrawn from the supernatant and the remainder was subjected to separation by dispersive solid phase extraction (SPE) using activated charcoal in Eppendorf tubes. The filtrate (F fraction) contained PRPP, while the nucleotides were eluted (E fraction) using 2% ammonium, 50% ethanol as eluent. The nucleotides and PRPP were quantified by applying F and E fractions on PEI-cellulose TLC plates and separating compounds by one-dimensional TLC using an optimized solvent containing 3 M ammonium formate (pH 2.4) and 0.7 M ammonium chloride (AFC buffer). Absolute nucleotide quantification was based on ATP counts from the R fractions separated using 0.85 M potassium phosphate (pH 3.4) as solvent and the total radioactive counts in the media sample. At an OD<sub>600</sub> of 0.3–1.0, we took 10 ml samples for RNA isolation of MG1363. The samples were directly transferred to ice-cold glassware, and the cells were spun down cold (2500 g for 10 min), washed in ice-cold 0.9% NaCl and stored at −20 °C. Samples for RNA and nucleotide pool determination were taken from two independent cultures grown a week apart.

**Isolation of RNA.** RNA was extracted by a method using phenol extraction. Four hundred microlitres of phenol equilibrated with 100 mM sodium acetate (pH 4.8) (phenol/acetate) was added to 0.5 ml of glass beads (106 mm, acid washed, Sigma), in a 1.5 ml screw-cap tube and mixed. The cell pellets were resuspended in 200 µl of an ice-cold solution of 0.3 M sucrose and 0.01 M sodium acetate and treated with diethylpyrocarbonate (DEPC). Two hundred microlitres of a DEPC-treated solution of 2% SDS and 0.01 M sodium acetate was added. The mixture was transferred to the tube with phenol and glass beads, which was shaken three times for 45 s at speed 4, with 1 min intervals, in a FastPrep FP120 (Savant) instrument for cell disruption and then placed on ice. The mixture was then incubated at 65 °C for 3 min, vortexed, placed in dry ice (3 min), and centrifuged for 5 min at 15 000 g. The upper phase was transferred to a new tube, and the following was performed twice: 400 µl of phenol/acetate was added, followed by vortexing, incubation at 65 °C for 3 min with two rounds of 1 s vortexing, cooling on dry ice for 3 min, centrifugation 5 min at 13 000 g, and transfer of the upper phase to a new tube. Four hundred microlitres of a 1:1 mixture of phenol/acetate and chloroform was added. The tube was centrifuged at 13 000 g for 5 min, and the upper phase was transferred to a new tube. RNA was precipitated by addition of 40 µl of 3 M sodium acetate and 900 µl 96% ethanol followed by brief vortexing. After being cooled on ice for 5 min, the tube was centrifuged at 13 000 g for 5 min at 4 °C. The pellet was washed with 500 µl 70% ethanol, pelleted by centrifugation, dried for 10 min in a vacuum centrifuge, and resuspended in 25 µl DEPC-treated water.

**Microarray analysis.** Transcriptomes were generated from seven samples from one culture and eight samples from another. The RNA was analysed using Agilent custom microarrays mapped to the *L. lactis* subsp. cremoris MG1363 genome (RefSeq: NC_009004.1) with five probes per gene. Images were analysed with an Agilent 2-micron scanner, and numerical values for features were calculated from the images with Agilent Feature Extraction software. The raw probe-level data were processed using the function ‘preprocess’ in the Bioconductor (Gentleman et al., 2004) package ‘preprocessCore’. Initial examination of the data revealed that the time points clustered in three groups: before, under, and after the shift in growth rate. Briefly, in the data sets of 15 time points, noise constituted by the expression values for each gene was smoothed out using a parallel regression model using the R package ‘gam’ (http://CRAN.R-project.org/package=gam) and the mean-centred log, expressions were analysed using principal component analysis (PCA), which revealed that the first three components explained 97% of the variance. Sparse PCA using the first three components was employed to lower the dimensionality, and for further analysis, genes outside the ellipsoid defined by K=0.999 in a chi-squared distribution, or equivalent to a Mahanalobis distance of 4, were chosen, resulting in 102 genes. The genes were hierarchically clustered. The microarray data are available at GEO with the accession number GSE52135.

**In vivo kinetics.** *In vivo* kinetic analysis of mRNA levels as a function of the PRPP level was performed using fitted mathematical descriptions of the curve forms: linear descriptions for the linear part, and fourth degree polynomial for the remaining part. The last data point before the sharp rise was included in both curves. The mRNA(t+t+c) function was plotted against the PRPP(t) function for t values from 0 to 102 min, and for c values from −8 to 2 min. The c value changes the response time for the mRNA levels relative to the PRPP levels, and the optimal c value was determined as the value that...
results in a homogeneous and unambiguous mRNA(PRPP) relation. The c value resulting in optimal timing of the purC curve was set to 0.

**Studies of xpt regulation.** Transcriptional fusions of the wild-type and mutant xpt promoters to a β-galactosidase reporter were constructed as follows. The promoter fragment was amplified from chromosomal DNA using the oligonucleotides MK466 (AAAAA-GCTTCATTGAAGGCGCAACTTCG) and MK398 (AAAAAGAT-CTCCGTCTGTATTAATGCGGTCTTC) and cloned in HindIII and BglIII sites (underlined) of pLB86 (Brøndsted & Hammer, 1999), and incorporated in the chromosome, yielding strain ML49. A mutant xpt promoter was created by overlap-extension PCR using further MK471 (ACGAGAAATTTTTCTCACCTGGGGCACAGATG) and MK472 (C-CTCCGTCTGTATTAATGCGGTC) and cloned in

**Rapid amplification of cDNA ends (RACE).** The transcription start site for the xpt-pbux operon was determined by 5′ rapid amplification of cDNA ends. cDNA was synthesized from chromosomal DNA using the primer MK451 (CCGTCGTATTAATGCGGTCTTC), a RACE primer (pGAAGAGAGGTTGAAATGGGCGTTTTG) was ligated, and the product was amplified using an anti-RACE primer (CGAA-AAGCGATTTCCACCTTG) and an internal primer MK450 (GTCGCCAGGGTAGAAGAAACG). The DNA was cloned using the TOPO TA kit (Invitrogen) and five clones were sequenced.

**RESULTS AND DISCUSSION**

**Depletion of an exogenous purine source induces transcription of purC and reduces the growth rate**

As an example of a specific temporal change in metabolism and gene expression, we examined exhaustion of a single exogenous substrate: the purine base hypoxanthine. The highest sampling rate should cover the period of transition, whereas fourth degree polynomials are used after 54 min.

**Changes in intracellular pools of nucleoside triphosphates (NTPs) and precursor PRPP during purine depletion**

A comprehensive experimental setup was designed to obtain genome-wide transcriptomic data from cultures of *L. lactis* strain MG1363, correlated with the intracellular pool sizes of nucleoside triphosphates. Multiple samples for nucleotide pool determination and RNA extraction were taken from two biologically independent cultures before and after the downshift illustrated in Fig. S1. The highest sampling frequency was around the shift. Samples for pool determinations were efficiently quenched and analysed for NTPs and PRPP (Jendresen *et al.*, 2011).

Fig. 1 shows the temporal development of the intracellular concentrations of ATP, GTP, CTP, UTP and the precursor PRPP. To enable extrapolation of the pool levels to intermediate time points, the level of each metabolite was identified, which resulted in purine exhaustion at an OD450 of 0.6. This concentration was subsequently used for the growth of four parallel cultures of the strain CBJ53 harbouring a purC-lacLM fusion, which permits easy analysis of purC transcription by a β-galactosidase assay. The optical densities and the corresponding total β-galactosidase activities in the four biological independent experiments were merged into a single plot (Fig. S2), and it showed a steep rise in the differential purc-lacLM synthesis rate (β-galactosidase/OD450) after an OD450 of 0.64, showing that the conditions were very reproducible and suitable for detailed studies. The synthesis rate after the shift was found to be 48 ± 3-fold higher than before the shift. This is in agreement with the previously obtained value of 52 for growth in the presence versus absence of a purine source (Jendresen *et al.*, 2012).
approximated with a linear function of the time of sampling, as the cultures should be in a steady state after having grown exponentially for more than eight generations. Strict balanced growth (Schaechter et al., 1958) is not possible for an auxotrophic organism as the medium composition changes during growth. The data points after the shift were fitted to a fourth degree polynomial (Fig. 1). Intracellular concentrations of PRPP and all NTPs were found to be rather constant or slightly rising until the shift in growth rate was detected. Upon the exhaustion of hypoxanthine, signified by the decreased growth rate, an immediate drop in ATP and GTP pool sizes was detected accompanied by an increase in PRPP. After approximately 10 min, ATP and GTP had reached their lowest concentrations at less than half of the pre-shift levels, whereas PRPP reached a maximum more than tenfold above the pre-shift level. During the next 15 min, the extreme intracellular concentration of PRPP returned to an intermediate level approximately threefold above the pre-shift level, while the ATP and GTP pools increased slightly towards new steady state levels. Apart from a slight drop in concentration immediately after purine depletion, concentrations of the pyrimidine nucleotides CTP and UTP were barely affected, and hence beyond significance for the physiology of the cell.

The correlation between the ATP and PRPP levels fits well with the hypothetical model for lowering of the PRPP level in Bacillus subtilis upon purine addition (Weng et al., 1995). The authors suggested that purine addition would lead to an increase in ADP, resulting in inhibition of the PRPP synthase through binding to its known allosteric ADP binding site.

Fig. 2. Genes responding to purine depletion. (a) Hierarchical clustering of responsive genes over 15 time points (identified using the time course in Fig. 1) revealed six distinct clusters: (1) encoding genes in the arginine and pyrimidine biosynthesis, (2) mainly consisting of ribosomal proteins, (3) nitrogen assimilation, (4) members of the PurR regulon, (5) clpE and hypothetical genes and (6) heat-shock proteins. Colours range from black (more than two log₂-fold below mean expression) to red (more than two log₂-fold above). (b) Expression is given as mean-centred log₂-transformed expression as a function of time.
From Fig. 1, it is clear that the PRPP level rises at the same time as the ATP level drops as a result of hypoxanthine depletion.

**Genome-wide transcriptional response to purine depletion**

After having established that purine depletion significantly induces both purC expression and PRPP pool size whereas ATP and GTP pools sizes are significantly reduced, a genome-wide transcriptomic analysis of RNA samples extracted from the cultures was performed. Fifteen RNA samples taken before, during and after purine depletion were analysed, using customized full genome Agilent microarrays.

After normalization of the data, hierarchical clustering of the most responsive genes in the transcriptome showed distinct behavioural patterns as illustrated in the heat map in Fig. 2(a). The genes could be divided into six clusters, whose distinct expression profiles are shown in Fig. 2(b). A list of all the genes assigned to the individual clusters can be found in Table S1. The most dramatically differing profiles are found between the highly responsive clusters 1 and 4 whose members are oppositely regulated after the shift. Patterns within cluster 2 and 5 show similarities to the patterns in cluster 1 and 4 respectively. However, unlike the latter, the mRNA levels in clusters 2 and 5 return to the pre-shift levels after 30 min.

Cluster 1 members are mostly genes involved in biosynthesis and transport of arginine – *argB, argE, argG, argH, gltS* – as well as genes related to pyrimidine biosynthesis, such as the *pyrEC* and *pyrKDbF-llmg_1108* operons.

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**Fig. 3.** Determination of *in vivo* kinetics. Relationship between temporal intracellular PRPP levels (a) and *purC* mRNA levels (b). After fitting of the linear section before purine deprivation to a linear curve, and the remaining datapoints to fourth degree polynomial relationships, the corresponding PRPP and *purC* mRNA levels were plotted for each time point from 0 to 102 min. (c) The effect of varying the timing between the PRPP and *purC* mRNA curves at 1 min before (grey triangles) and 1 min after (grey diamonds) around the optimal timing (shown in black). (d) The result of the optimal timing on the temporal behaviour of the two curves.
Although the pyrimidine and arginine biosynthesis share carbamyl phosphate as substrates, the genes of the two pathways are members of different regulons. The \( \text{argCJDBF, argGH, and gltS-argE} \) operons are regulated by the ArgR and AhrC regulators in response to the arginine availability (Larsen et al., 2004), while the \( \text{pyrEC and pyrKDBF-llmg\_1108} \) operons are regulated by the PyrR regulator (Martinussen et al., 2001). The PyrR homologue

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\begin{align*}
\text{purC mRNA level} & \quad \text{purS mRNA level} & \quad \text{purQ mRNA level} \\
\text{purL mRNA level (+2 min)} & \quad \text{purF mRNA level (+2 min)} & \quad \text{llmg\_0978 mRNA level (+4 min)} \\
\text{purD mRNA level (+1 min)} & \quad \text{llmg\_0998 mRNA level} & \quad \text{purE mRNA level} \\
\text{purK mRNA level (+1 min)} & \quad \text{purM mRNA level} & \quad \text{purN mRNA level}
\end{align*}
\]

**Fig. 4.** In vivo PRPP kinetics of the genes of the \( \text{purCSQLF, purDEK} \) and \( \text{purMN} \) operons of the PurR regulon. Levels of mRNA from curves fitted to experimental data points (gene designation is noted above each figure) are plotted as functions of PRPP levels from the curve shown in Fig. 3(a). The optimal timing for the \( \text{purC-PRPP} \) relationship was used for the plots, unless otherwise noted in parentheses after the gene designation.
in *B. subtilis* has been shown *in vitro* to respond to the UMP and PRPP availability (Lu & Switzer, 1996).

The expression of the genes in cluster 2 decreases upon purine depletion and it returns to approximately the pre-shift levels. This cluster contains mostly genes related to ribosome function, which have been shown to be growth rate regulated (Dressaire *et al.*, 2008).

Cluster 3 contains three genes: *amtlB* and *glnB* which are involved in nitrogen metabolism, and *hprT* which encodes one of two isoforms of the G/Hx PRTases that synthesize GMP and IMP from guanine and hypoxanthine, respectively (Kilstrup *et al.*, 2005). The second isoform is encoded by the *hpt* gene, and is apparently not responding to purine depletion.

Genes from cluster 4 are all transcribed from promoters involved (Kilstrup *et al.*, 2005). The second isoform is encoded by the *hpt* gene, and is apparently not responding to purine depletion.

Cluster 5 contains the *clpE* chaperone gene and three genes of unknown function.

The four genes that together constitute cluster 6 are slightly induced during the shift, but they subsequently drop below the pre-shift levels. These genes belong to the *groEL* and *dnaK-grpE-hrcA* operons and constitute the HrcA heat shock regulon (Arnau *et al.*, 1996; van Asseldonk *et al.*, 1993; Hansen *et al.*, 2001; Kim & Batt, 1993). A sudden decrease in purine nucleotides is likely to influence translation and cause translational stress.

**In vivo PRPP kinetics of PurR regulated genes**

The expression of the purine biosynthetic genes is regulated by PurR in a feed-forward manner, and a dependence on the presence of the precursor PRPP has been shown for the *L. lactis* regulator (Jendresen *et al.*, 2012; Kilstrup & Martinussen, 1998; Kilstrup *et al.*, 1998). However, no kinetic data have yet been published for PurR activity in this organism. In *B. subtilis* the DNA binding properties have been determined *in vitro* as a function of the PRPP concentration (Bera *et al.*, 2003). We have reported that PurR binds to a PurBox under both activating and non-activating conditions (Kilstrup & Martinussen, 1998), so measuring PRPP kinetics of PurR activity *in vitro* would involve a transcriptional assay. A more direct approach would be to measure PurR activity as a function of the PRPP concentration *in vivo*. This could be achieved by measuring the transcriptional level of different genes of the PurR regulon, and correlating the activity to the PRPP metabolite levels. Because the causal agent (PRPP level) and the effect (resulting mRNA levels) are separated by the

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**Fig. 5.** *In vivo* PRPP kinetics of genes from the *pyrEC*, and *pyrKDbF-llmg_1108* operons of the PyrR regulon. Levels of mRNA from curves fitted to experimental data points (gene designation is noted above each figure) are plotted as functions of PRPP levels from the curve shown in Fig. 3(a). The optimal timing for the *purC*-PRPP relation was used for the plots, unless otherwise noted in parentheses after the gene designation.
transcriptional elongation time, the correlation had to be adjusted with respect to time. Fig. 3(a) shows the response curves for the PRPP level (data from Fig. 1), and Fig. 3(b) the levels of purC mRNA from the transcriptome analysis. The data points sampled before hypoxanthine depletion were fitted to a linear function and the remaining data to a fourth degree polynomial. The corresponding values of PRPP and purC mRNA that were calculated from the fitted polynomial formula were plotted in one-minute intervals from time 0 to time 102 min. By sliding the mRNA levels relative to the PRPP levels, we obtained the curves in Fig. 3(c). At the optimal timing, the correlation assumed a homogeneous and unambiguous curve of purC mRNA levels as a function of the PRPP level. When the timing of the curves was varied by one minute in either direction, the relationship disintegrated. Fig. 3(d) shows the resulting temporal relationships between PRPP levels and time corrected purC mRNA levels. The sharp rise in the curves of PRPP and mRNA levels (Fig. 3c) prevented us from accurately describing the function between 2 and 4 units during this fast rising transition period, which lasted for less than three minutes. The last of our time points in the linear phase in our dataset was used as the transition point. We suppose that the curves would have had a smooth, monotonic transition of purC mRNA levels, if the extra data had been available, and we omitted the fitted data in this area in the following calculations. We examined how each cluster of genes responded to the change in PRPP concentration.

The same kinetic relationship between mRNA and PRPP levels was shared for all the genes in the purCSQLF-llmg_0978

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**Fig. 6.** Structures of the xpt riboswitch and terminator. The transcription start point was determined by 5’ RACE, and riboswitch and transcription terminator structures (a) were modelled after Mandal et al. (2003), with the sequence truncated after a putative terminator located before the xpt gene. A large alternative antiterminator structure can be formed in absence of a stabilized riboswitch (b). P1, P2, P3 are helices found in all purine riboswitches, and P4 is a stem that is formed in both alternative structures. The xpt start codon is underlined and the two nucleotides indicated by a black box were mutated to AA, disrupting the terminator, but not the antiterminator.
operon (Fig. 4) when the purL and purF genes were allowed two additional minutes and llmg_0978 four additional minutes to respond to PRPP, which is in accordance with the expected delay considering the elongation speed of the RNA polymerase. This result further signifies that a multiple-gene operon can be identified using the present method. Overall, the PRPP kinetics of the PurR activation appears similar for the genes in the purD-llmg_0998-EK operon (Fig. 4). However, the first and third of the genes have to be time adjusted by one minute to achieve kinetic consistency.

Whereas the pur operons appear to follow sigmoid kinetics, the purine gene guaC and the pbuO gene for a purine transporter (Figs S3 and S4) appear to follow normal Langmuir saturation kinetics. The kinetics of the genes involved in folate one-carbon metabolism, the folD-xseAB operon, and the fsh and glyA genes (Fig. S3) likewise appear to follow normal saturation kinetics, but they have a long response time compared to the pur operons. Unusual behaviour is observed for some of the genes in the phnDCB-llmg_0315-cpdC-llmg_0317 operon (Fig. S5). The first gene shows normal kinetics towards PRPP, while the rest show higher mRNA levels than expected, as expression was continuously increasing after the shift. This could be explained by RNase dependent processing of the mRNA after the phnD gene, resulting in normal half-life of the phnD mRNA but increased half-life of the following part. An opposite behaviour is found for the hprT gene, in the hprT-purH-llmg_0995 operon, where the last two genes show normal PRPP dependence, but the hprT gene show lower mRNA levels than expected. This difference in mRNA can be explained by differential regulation, and indeed there is a separate PurR-regulated promoter present between hprT and purH (Jendresen et al., 2012).

**In vivo PRPP kinetics of PyrR regulated genes**

When mRNA levels for non-PurR regulon genes were analysed, it was impossible to obtain homogeneous and unambiguous curves showing PRPP dependent kinetics. Exceptions to this behaviour were found among the pyr genes in cluster 1. As seen in Fig. 5, the mRNA levels from genes of the pyrEC and the pyrKDbF-llmg_1108 operons responded in a consistent way showing an inverse correlation with PRPP concentrations. Interestingly, in *B. subtilis*, the PyrR regulator that controls the genes is known to respond to the UTP or UMP concentration by facilitating transcriptional attenuation of the pyrimidine biosynthetic operon, leading to termination of transcription. PRPP and GTP have been shown to counteract the binding of UMP to PyrR *in vitro* (Bonner et al., 2001), but here there was a negative correlation between PRPP and PyrR regulon mRNA levels. The changing of pyrimidine and purine NTPs are balanced by nucleoside diphosphate kinase and it is likely that there was significant build-up of UMP and UDP during the shift.

**Confirmation of predictions from in vivo kinetics: the unusual PRPP kinetics of xpt-pbuX PurR activation is due to overlapping riboswitch control**

A particular PurR regulated promoter showed no correlation of transcript levels with the PRPP level. The mRNA levels of both genes of the xpt-pbuX operon (Fig. S4) were much higher after the shift than expected for normal PurR kinetics (Fig. 4). This could theoretically be due to an exceptionally long mRNA half-life of the transcript. However, a putative riboswitch structure immediately following the PurR regulated promoter has been suggested (Mandal et al., 2003), and to confirm the predictive power of the *in vivo* kinetic analysis, the overlapping riboswitch dependent regulation of the xpt promoter was experimentally verified. We identified the transcriptional start site, which corresponded to the PurBox-promoter previously described (Jendresen et al., 2012). Fig. 6 shows the predicted riboswitch and the suggested terminator and antiterminator. We constructed a transcriptional reporter fusion to the xpt promoter. The expression pattern was compared to the expression from a promoter region in which the riboswitch-associated terminator was mutated, thereby removing riboswitch-mediated regulation. As seen in Fig. 7, the expression level from the terminator mutant was fivefold higher than the wild-type level, which shows that the terminator is active in the absence of purine supplements. Interestingly, the wild-type fusion responded strongly to adenine, which is known to affect PurR dependent gene expression, as well as to guanosine, which does not affect PurR dependent expression (Klstrup & Martinussen, 1998). In contrast, the riboswitch mutant responded strongly to adenine, but any trace of PurR independent

![Fig. 7. Effect of disruption of the terminator in the xpt 5′ untranslated region. The putative terminator structure, which was hypothesized to be part of the riboswitch regulation of the xpt-pbuX operon, was mutated as indicated in Fig. 6. The expression levels (Miller units) were measured from transcriptional fusions to a β-galactosidase reporter gene, and the mutant lost regulation specifically to guanosine but not adenine, a regulation which is mediated through PurR. Error bars represent so of three independent experiments.](http://mic.sgmjournals.org)
regulation by guanosine was absent. This shows that the putative riboswitch structure is functional, and that its function results in dual purine regulation of xpt and pbuX expression. This indeed indicates that the riboswitch regulation is the cause of the atypical PRPP kinetics for the promoter. As the effect of adenine could be singled out, it furthermore indicates that the PRPP-mediated regulation acts through ADP mediated inhibition of PRPP synthase by adenine nucleotides, as is the case in B. subtilis.

Conclusions

Here, we have characterized the in vivo response to a metabolic change, and linked the concentration of inducer to transcriptional activation. The patterns of regulation could identify operons as well as situations where auxiliary regulation was taking place. We are currently preparing further experiments for in vivo regulatory kinetics using novel detection methods (Magdenoska et al., 2013). As sequencing costs are decreasing rapidly and metabolomics is becoming more accessible, the principles of in vivo regulatory kinetics presented becomes a means to identify, describe and understand mechanisms of gene regulation on a genome scale level.

ACKNOWLEDGEMENTS

Marzanna Pulka-Amin is thanked for help with RNA isolation, transcriptional fusions and β-galactosidase measurements. Lene Christiansen is thanked for her contributions in running microarrays.

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Edited by: J. Kok