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# Disparity between changes in mRNA abundance and enzyme activity in *Corynebacterium glutamicum*: implications for DNA microarray analysis

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**Abstract** The relationship between changes in mRNA abundance and enzyme activity was determined for three genes over a span of nearly 3 h during amino acid production in *Corynebacterium glutamicum*. Gene expression changes during *C. glutamicum* fermentations were examined by complementary DNA (cDNA) microarrays and by a second method for quantitating RNA levels, competitive reverse transcriptase-PCR (RT-PCR). The results obtained independently by both methods were compared and found to be in agreement, thus validating the quantitative potential of DNA microarrays for gene expression profiling. Evidence of a disparity between mRNA abundance and enzyme activity is presented and supports our belief that it is difficult to generally predict protein activity from quantitative transcriptome data. Homoserine dehydrogenase, threonine dehydratase, and homoserine kinase are enzymes involved in the biosynthesis of L-isoleucine and other aspartate-derived amino acids in *C. glutamicum*. Our data suggest that different underlying regulatory mechanisms may be connected with the expression of the genes encoding each of these three enzymes. Indeed, whereas in one case the increases in enzyme activity exceeded those in the corresponding mRNA abundance, in another case large increases in the levels of gene expression were not congruent with changes in enzyme activity.

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## Introduction

*Corynebacterium glutamicum* is used in the commercial production of amino acids, primarily glutamic acid and lysine. High-level amino acid biosynthesis places physiological stress upon the cell as it draws resources from many different biosynthetic pathways (Lessard et al. 1999). To understand how *Corynebacterium* accommodates these demands, research in recent years has sought to elucidate the overall regulatory processes that coordinate the cell's physiology during amino acid production. We have recently applied DNA microarraying techniques to the study of changes in the global transcriptome patterns during amino acid production. The results showed that small microarrays comprising as few as about 50 genes can provide statistically robust data sets (Loos et al. 2001).

DNA microarrays enable simultaneous measurement of relative transcript levels for many thousands of genes in a single experiment. This expression profiling is useful for examining the dynamics of global transcriptional regulation on a scale heretofore unimaginable. Our hope is that such gene expression fingerprints can be used to decipher genetic networks and regulatory mechanisms that define a particular biological pathway, physiological state, or phenotype, such as high productivity in a fermentor.

To fully describe the state of any biological system, it is essential to determine not only one but all of its components (i.e. transcriptome, metabolome, and proteome). A comprehensive understanding of the underlying networks leading to a distinct phenotype relies on the combination of the different factors contributing to it. Therefore it remains important to understand where the new DNA microarray technique (and its generated transcriptome data) fit within the framework of existing techniques if we are to fully unlock its potential. Though

often inferred, the quantitative aspect of DNA microarrays has not been fully investigated and little work has been published validating data obtained using DNA microarrays with other conventional methods. Therefore, validation of the performance of DNA microarrays is still essential for the successful application of this method.

It is also wrong to assume that responses at the mRNA level reflect the response at the protein level or at the level of active enzyme, i.e. that there is a static correlation between levels of mRNA, protein, and active enzyme. Recently, researchers have begun to examine the relationship between transcriptome and proteome on a large scale (Gmuender et al. 2001; Yoshida et al. 2001). However, few studies have quantitatively examined the correlation between message and protein abundance. Gygi et al. (1999) compared the relative protein and mRNA abundances of more than 150 targets in yeast. Protein data were obtained by quantitative 2D gel electrophoresis, and serial analysis of gene expression (SAGE) was used for quantitation of mRNA levels. Anderson and Seilhamer (1997) examined the relationship of mRNA and protein levels in human liver for about 20 different targets. They, too, quantitated protein abundance by 2D gel electrophoresis, but used transcript image methodology, a relatively low-throughput method, for transcriptional quantitation. Both studies showed some evidence for the existence of a disparity between the relative expression levels of mRNAs and their corresponding proteins. Ritter et al. (2002) used quantitative RT-PCR and immunohistochemistry to confirm DNA microarray results. Although these studies addressed the quantitative correlation between transcriptome and proteome, they did not address the relationship between expression and enzyme activity. The examination of enzyme activity is key considering the complex regulatory mechanisms for gene expression that occur at both post-transcriptional and post-translational levels.

In this work, the industrially important prokaryote *C. glutamicum* was used as a model organism to address the question of correlations between transcriptome and proteome. In contrast to existing studies, the relative levels of mRNAs were compared to the proteins' enzyme activities rather than to their abundances. Furthermore, rather than analyzing a single time point, the relative changes in both mRNA and enzyme activity over time were examined. Additionally, the question of accuracy of measurements of mRNA abundance obtained by DNA microarray analysis was addressed by using a second method for measuring relative mRNA levels. We conclude that data about the transcriptome generated with DNA microarrays is valuable but must be augmented with other data to fully describe and define biological systems. To our knowledge, this is the first study in which microarrays have been used to compare changes in mRNA and enzyme activity levels under different physiological conditions.

## Materials and methods

### Strains, plasmids, and media

*Corynebacterium glutamicum* strain ATCC 21799 (American Type Culture Collection, Manassas, Va.) is an aminoethylcysteine-resistant, L-leucine auxotroph, lysine-producing strain derived from *C. glutamicum* strain ATCC 13869. Expression plasmid pAPE20 (Guillouet et al. 2001), a broad-host-range vector conferring kanamycin resistance, carries copies of *C. glutamicum* genes encoding threonine dehydratase (*ilvA*, GenBank accession no. L01508), homoserine kinase (*thrB*, Y00546) and a feedback-resistant form of homoserine dehydrogenase ($hom^{dr}$, Y00546, (Archer et al., 1991)). The plasmid-borne *ilvA* and *thrB* genes are expressed under the control of IPTG-inducible promoters (P*trc* and P*tac*, respectively), whereas the $hom^{dr}$ gene is preceded by its native promoter. *C. glutamicum* fermentation medium was prepared as described (Guillouet et al. 1999), with the exception that the glucose concentration was 40 g/l.

### Fermentations

Batch fermentations(3 l) of *C. glutamicum* ATCC 21799 (pAPE20) were carried out as described by Guillouet et al. (2001). The cultures were grown at 30 °C with an aeration rate of 1 vvm and an agitation rate of 1,500 rpm. The pH was maintained at 7.5 with ammonium hydroxide and hydrochloric acid solutions. Methods used for determining biomass, sugars, and amino acids were described by Guillouet et al. (1999).

### Enzyme assays

Enzyme assays were carried out with cell-free crude extracts prepared by the method described by Guillouet et al. (1999). Spectrophotometric assays for determining activity of homoserine dehydrogenase (HDH; EC 1.1.1.3), threonine dehydratase (TD; EC 4.2.1.16) and homoserine kinase (HK; EC 2.7.1.39) were carried out as described by Guillouet et al. (1999, 2001).

### RNA preparation

Cell culture samples were flash-frozen in liquid nitrogen and then stored at −80 °C. Prior to RNA extraction, samples were equilibrated at −20 °C overnight. Frozen bacterial cells were thawed on ice, harvested by centrifugation for 5 min at 5,000 *g* at 4 °C, and resuspended in cold RLT lysis buffer containing guanidium thiocyanate/$\beta$-mercaptoethanol (RNeasy Midi kit, Qiagen, Valencia, Calif.) to a final concentration of 15 g dry cell weight (DCW)/l. Twelve ml of this cell suspension were mixed with 12 ml of cold 0.1 mm zirconia/silica beads (BioSpec Products, Bartlesville, Okla.). Cells were mechanically disrupted at 4 °C for eight cycles of 30 s spinning separated by rest cycles of 30 s in a Bead-Beater (BioSpec Products). Subsequently, RNA was purified using the RNeasy Midi kit (Qiagen) including on-column DNA digestion. RNA was quantified using the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, Ore.). Typically, 1–2 mg RNA were recovered from a 100-ml culture sample with a biomass of 1.5 g DCW/l by this method. Each RNA sample was examined by agarose gel electrophoresis, which revealed distinct ribosomal RNA bands with little evidence of degradation. All samples reported $OD_{260}/OD_{280}$ ratios in the range of 1.8, as measured in unbuffered water.

### cDNA microarrays

cDNA microarrays were fabricated using PCR products representing a set of 72 *C. glutamicum* open reading frames (ORFs). Most of the printed cDNAs represent PCR products that have been

described by Loos et al. (2001). In addition to *Corynebacterium* ORFs, PCR products representing two ORFs of *S. cerevisiae* (YAL058C-A and YAL047C) were printed and served as internal controls of the data normalization process. PCR products (approximately 0.2 mg/ml in 50% DMSO) were printed in triplicate onto amino-silane-coated slides (CMT-GAPS II Coated Slides, Corning, N.Y.). Protocols for processing slides after printing and for reverse transcription and labeling were adapted from methods described by the laboratory of Patrick Brown (http://cmgm.stanford.edu/pbrown). To denature the RNA, 20 µg of total *C. glutamicum* RNA, 0.01 µg in vitro transcribed yeast RNA (controls), and 1 µg random DNA hexamers (Roche Diagnostics) were incubated for 10 min at 70 °C and chilled on ice for 2 min. From this RNA, a reaction mix was prepared containing 25 U RNase inhibitor, 400 U SuperscriptII reverse transcriptase (GibcoBRL, Bethesda, Md.), dATP, dCTP, dGTP (0.5 mM each), dUTP (0.2 mM), DTT (10 mM) and either Cy3- or Cy5-labeled dUTP (2.5 mM) (NEN, Boston, Mass.). The resulting mixture was incubated for 10 min at 25 °C (annealing) and subsequently for 2 h at 42 °C (reverse transcription). For RNA digestion, 2 U RNaseH (Roche Diagnostics) and 0.5 µg RNase (Roche Diagnostics) were added to each reaction and incubated for 1 h at 37 °C. Corresponding cDNA samples (Cy3- and Cy5-labeled cDNA) were combined and purified using a Qiaquick PCR purification kit (Qiagen). After elution in the supplied Tris buffer, the slightly colored solution was diluted with 400 µl of water and then concentrated to approximately 25 µl in a Centricon YM-30 (Millipore , Bedford, Mass.). Samples were dried for 20 min under vacuum in a microcentrifuge. For each time point, two hybridization experiments were carried out. "Control RNA" was prepared by pooling equal amounts of total RNA, as measured by the RiboGreen assay (Molecular Probes), isolated from three shake-flask cultures harvested before IPTG induction (1.65 g DCW/l), 3.25 h after induction (4.25 g DC)/l), and 7.25 h after induction (7.8 g DCW/l), respectively. Whereas in the first experiment, the "control RNA" was reverse-transcribed and labeled using the fluorescent dye Cy3-dUTP, and the "experimental RNA" using the Cy5-dUTP dye, in the second experiment the dyes were swapped and the samples labeled inversely to correct for nonlinear signal.

Protocols for array hybridization were adapted from Hegde et al. (2000). To the labeled cDNA from the previous step, 15 µl of a solution containing formamide (25%), SSC (5×) (1× SSC in 0.15 M NaCl plus 0.015 M sodium citrate), SDS (0.1%) and herring sperm DNA (1.3 mg/ml) were added, boiled for 2 min, and quickly chilled on ice. The slide was positioned in an ArrayIt hybridization cassette (TeleChem International, Sunnyvale, Calif.) and a lifter slip (Erie Scientific, Portsmouth, N.H.) placed over the array. Hybridization solution was pipetted under the lifter slip and the sealed hybridization chamber was submerged in a 42 °C water bath for 12–16 h. Next, the slides were washed in SSC (1×) containing SDS (0.03%) for 5 min, then in SSC (0.2×) for 2 min, and last in SSC (0.05×) for 15 s. Slides were dried with nitrogen gas and scanned within the next 6 h using an ArrayWoRx CCD scanner (Applied Precision, Issaquah, Wash.) to measure fluorescence of the Cy3- and Cy5-labeled cDNAs bound to the DNA microarray. Statistical analysis was carried out using the methods described by Loos et al. (2001).

### Competitive RT-PCR

Target sequences 200 bp in length within the open reading frames (ORFs) of the three targets (*hom*, *ilvA*, *thrB*) were chosen for amplification. Exogenous, homologous RNA standards of 215 nucleotides, carrying a 15-nucleotide insertion compared to the native gene, were created for coamplification. The sequence 5'-aaatttatatatata-3' was inserted into clones of each of the three genes and 215-bp DNA fragments were generated by mutational PCR. The resulting PCR products were cloned into plasmids downstream of a T7 promoter. Linearized forms of these plasmids were used as template DNA for in vitro transcription. Resulting RNA transcripts were purified (RNeasy Mini Kit, Qiagen), quantified using the RiboGreen RNA Quantitation Kit (Molecular Probes) and used as gene-specific, exogenous, homologous RNA standards during competitive RT-PCR. For RT-PCR, a single-tube one-step kit was used (Qiagen). For each experiment a series of seven reaction tubes was run in parallel. Each contained identical amounts of *C. glutamicum* total RNA (between 50 and 500 ng) plus the gene-specific in-vitro-transcribed RNA standard in increasing amounts from tube to tube (1, 2, 4, 8, 16, 32 and 64 pg). Cycling conditions (Robocycler Infinity, Stratagene, La Jolla, Calif.) were as follows: (1) reverse transcription for 30 min at 50 °C; (2) DNA polymerase activation for 15 min at 95 °C; (3) denaturing for 60 s at 94 °C; (4) annealing for 45 s at 61 °C; (5) extension for 60 s at 72 °C; (6) final extension for 5 min at 72 °C. Steps 3–5 were repeated for a total of 15 cycles. Controls to detect potential DNA contamination were run with each experiment. RT-PCR products were analyzed by gel electrophoresis on 8% polyacrylamide gels in 1× TBE. After staining with ethidium bromide, gels were scanned using a fluorescence imaging system (Molecular Dynamics, Sunnyvale, Calif.) with an excitation wavelength at 526 nm and an emission wavelength at 605 nm. Scanned images were analyzed using the image analysis software tool ImageQuant (Molecular Dynamics).

## Results

Changes in gene expression and enzyme activity and their correlation under different physiological conditions were examined in batch fermentations of *C. glutamicum* ATCC 21799 (pAPE20). In this strain, overexpression of three genes encoding homoserine kinase (*thrB*), threonine dehydratase (*ilvA*) and a feedback-insensitive homoserine dehydrogenase ($hom^{dr}$) diverts carbon destined for lysine synthesis (as occurs in the parent strain) toward isoleucine (Guillouet et al. 2001). In plasmid pAPE20, *ilvA* and *thrB* are expressed under the control of IPTG-inducible promoters, whereas $hom^{dr}$ is preceded by its native promoter. These three genes were chosen for this study

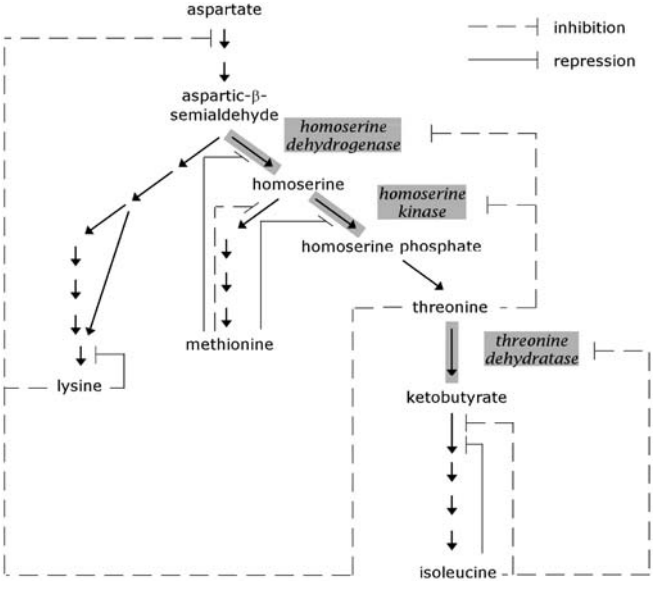


**Fig. 1** The biosynthesis and regulation of aspartate family amino acids in *Corynebacterium glutamicum*. Gene expression and enzyme activity studies were carried out for homoserine dehydrogenase (*hom*, HDH), homoserine kinase (*thrB*, HK) and threonine dehydratase (*ilvA*, TD)

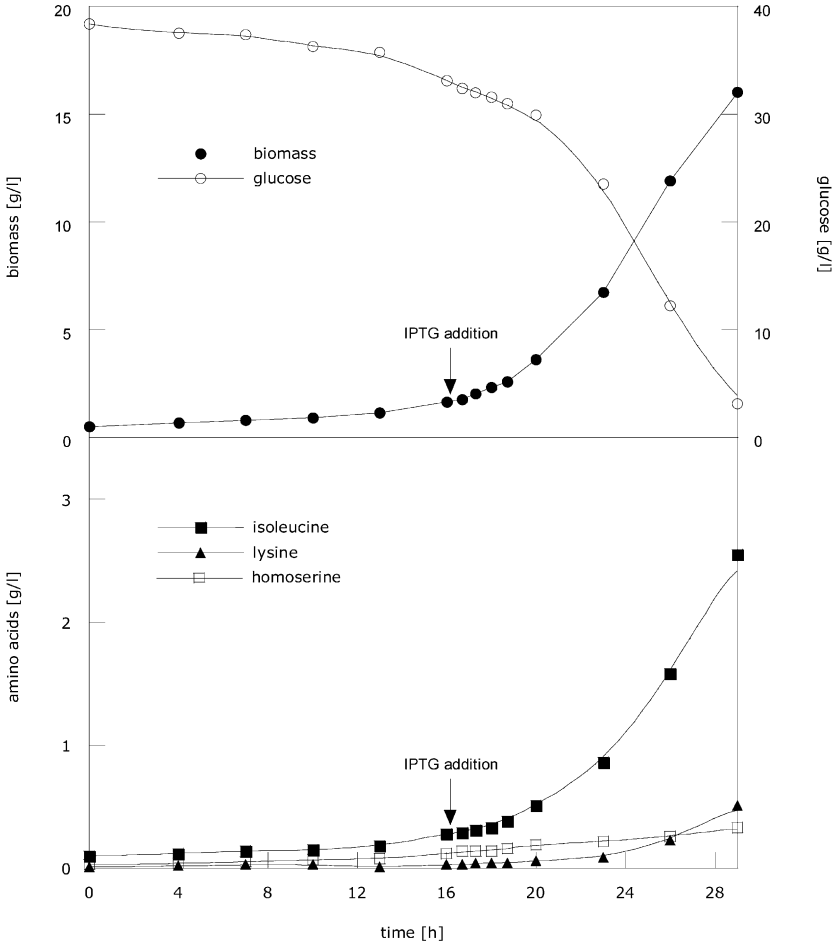


**Fig. 2** *C. glutamicum* ATCC 21799 (pAPE20) batch fermentations. Kinetics of growth, substrate consumption (*top*), and amino acid production (*bottom*). For analysis of gene expression and enzyme activity, five samples in 40-min intervals were harvested, starting at 16 h (immediately prior to the addition of IPTG)

because their products can be assayed enzymatically and their enzyme activities under defined conditions have already been investigated (Guillouet et al. 2001). Furthermore, they are all directly involved in the biosynthesis of isoleucine and other amino acids of the aspartate family (Fig. 1). The changes in gene expression and enzyme activity were analyzed over a time frame of five consecutive 40-min intervals during the early exponential growth phase. mRNA levels were measured independently by two different methods: cDNA microarrays and competitive RT-PCR. Enzyme activities were measured using established spectrophotometric assays. To compare the different data sets, the activities were normalized with respect to the first sampling point, in order to control for variation in the lag phase, and expressed in -fold changes over time.

## Fermentations

*C. glutamicum* ATCC 21799 (pAPE20) was grown in duplicate 3-l batch fermentations in the described fermentation medium. IPTG (150 mg/l) was added when the biomass reached 1.5 g DCW per liter. Kinetics of growth, substrate consumption, and amino acid production are given in Fig. 2. This strain grew exponentially with a maximum specific growth rate of 0.21±0.02 $h^{-1}$. The glucose to biomass conversion yield was 0.45±0.05 g DCW/g glucose and the glucose to isoleucine conversion yield was 0.07±0.01 g Ile/g glucose. The strain produced mainly isoleucine at a final concentration of 2.72±0.76 g/l. Final concentrations of lysine and homoserine were low, 0.56±0.21 g/l and 0.28±0.04 g/l. No threonine was detected in the medium. The results from these fermentations were consistent with those reported by Guillouet et al. (2001). Five samples taken in consecutive 40-min intervals were harvested from each fermentation for analysis of both gene expression and enzyme activities. The first sampling point was when the biomass reached 1.5 g DCW/l (at 16 h), then IPTG was added to the culture. By the last sampling point (160 min later), the biomass had reached a value of 2.8 g DCW/l.

## mRNA analysis

Changes in mRNA expression of *hom*, *ilvA* and *thrB* were analyzed using cDNA microarrays and independently by a second method, competitive RT-PCR (Raeymaekers 2000). While each of the three genes showed a different

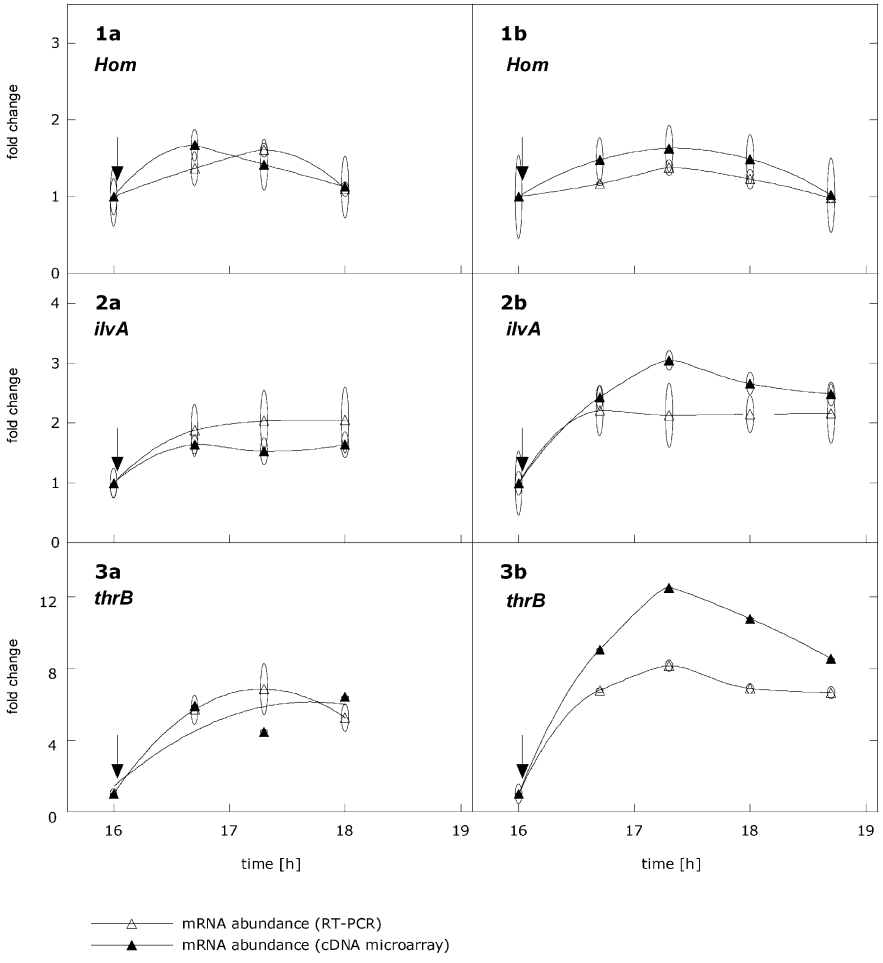


**Fig. 3** Independent measurement of mRNA levels by DNA microarray and competitive reverse transcriptase-PCR (RT-PCR). Relative mRNA abundances over time during two fermentations (**a**, **b**) of *1* homoserine dehydrogenase, *2* threonine dehydratase, *3* homoserine kinase. For comparison, each data set was normalized with respect to the first time point. *Ovals* standard deviations for each data point, *arrows* time of IPTG addition

pattern of mRNA abundance over time, these patterns were conserved in both fermentations (Fig. 3). The smallest changes over time in mRNA transcript levels were observed for homoserine dehydrogenase (*hom*). mRNA abundance of the threonine-dehydratase-encoding *ilvA* gene increased slightly over the first three time points, remaining fairly constant thereafter (Fig. 3). The largest increase in mRNA abundance was observed for the homoserine-kinase-encoding *thrB* gene. RT-PCR data showed a steep increase in mRNA abundance, peaking after 80 min, with a subsequent decrease and leveling off for the remaining time points analyzed (Fig. 3). Analysis by DNA microarrays suggested slightly different patterns of *thrB* expression in the two fermentations (Fig. 3). In the first fermentation, the magnitude of *thrB* mRNA levels measured was similar using both methods, but there were some differences in the pattern observed (Fig. 3). In the second fermentation, the pattern of mRNA abundance as measured by cDNA microarray was similar to that measured by RT-PCR but differed in magnitude of response. A combined dataset of mRNA abundance was constructed as a weighted average of the microarray and RT-PCR data for each gene and fermentation. This mRNA data set was then analyzed in comparison with subsequent enzyme activity data (Fig. 4).

## Comparison of mRNA abundance and enzyme activity

For the same three genes, enzyme activities were determined during both fermentations (Fig. 4). The results from these enzyme activity measurements were in agreement with those reported by Guillouet et al. (2001). Whereas both methods for measuring gene expression (competitive RT-PCR and cDNA microarrays) produced similar expression patterns for each of the three genes (*hom*, *ilvA*, *thrB*), significant differences were seen when comparing mRNA levels to the levels of enzyme activities found for each target (Fig. 4). The best correspondence between changes in mRNA abundance and enzyme activity was observed for homoserine dehydrogenase (*hom*, HDH), in which mRNA abundance and enzyme activities showed changes that were similar in profile and scale. A different relationship was observed for threonine dehydratase (*ilvA*, TD), in which increases in enzyme activity strongly exceeded those in mRNA abundance. *ilvA* mRNA levels moderately increased after adding IPTG and then stayed constant. Enzyme activity analysis, however, showed an increase in TD activity that exceeded the mRNA response by several fold. A third pattern emerged for homoserine kinase (*thrB*, HK). Here, large increases in the levels of mRNA transcripts were not

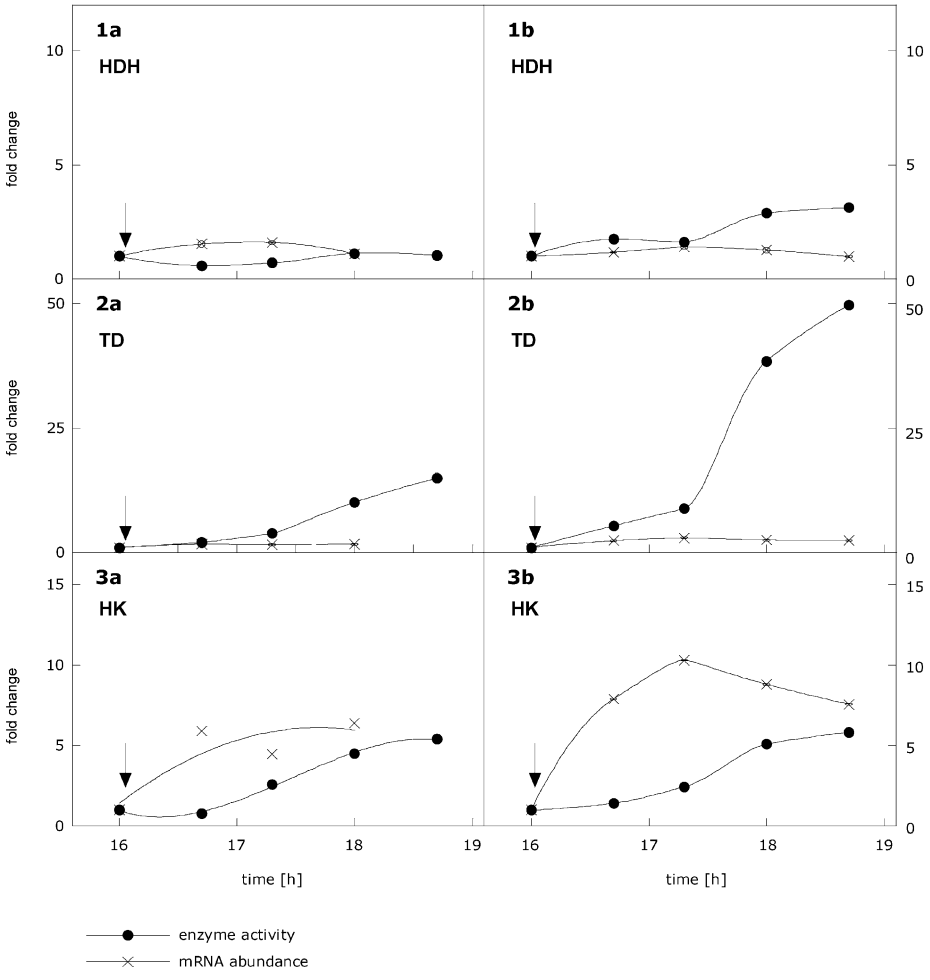


**Fig. 4** Correlation of mRNA and enzyme activity changes over time during two fermentations (**a**, **b**) of *1* homoserine dehydrogenase (*HDH*), *2* threonine dehydratase (*TD*), and *3* homoserine kinase (*HK*). Total RNA and protein preparations were each prepared from aliquots of the same culture samples. For comparison, all data sets were normalized with respect to the first time point. mRNA abundance is represented as a weighted average of the DNA microarray and competitive RT-PCR data presented in Fig. 3. *Ovals* standard deviations for each data point, *arrows* time of IPTG addition

congruent with the increases in enzyme activity. Levels of *thrB* mRNA showed an increase over the first three time points of analysis, followed by a decrease over the remainder of the fermentation. HK activity, however, showed a small but steady increase over the same time course.

## Discussion

DNA microarrays are currently the most powerful tools for performing genome-wide expression profiling for thousands of genes in a single experiment. They offer the ability to study global regulatory processes on the transcriptome level and are becoming an important addition to the metabolic engineering toolbox. Metabolic engineering of the industrially important microorganism *C. glutamicum* seeks to improve strain properties towards higher production of various amino acids. Quantitative description of physiological control under different conditions provides the rational basis for metabolic engineering. Models based solely on the description of the transcriptome severely simplify the in vivo state and neglect potential post-transcriptional and post-translational regulatory mechanisms affecting metabolite fluxes.

While DNA microarray techniques have reached broad acceptance and offer advantages such as high throughput and sophisticated data mining, it is still important to appreciate the strengths and limitations of microarray data. It has been demonstrated that microarray data are statistically significant (Lee et al. 2000; Loos et al. 2001; Tusher et al. 2001). The issue of whether the data are biologically significant is being addressed in several ways: through biological replicate samples, confirmation of results with other analytical techniques and, most informatively, by combining these data with other information about the state of the cell.

To assess the significance and reproducibility of changes in gene expression it is important to use true biological duplicates. Meticulously controlling culture conditions, such as with the use of a bioreactor, is essential for duplicating physiological states with a minimum of errant variation that may confound the interpretation of targeted perturbations. To determine whether DNA microarray data accurately measure relative mRNA levels, we measured relative changes in mRNA abundance by DNA microarrays and by a second method, competitive RT-PCR. These two independent methods of quantitation yield similar results. This further validates DNA microarrays as a useful tool for

determining relative mRNA levels (Baker et al. 2001). Additionally, the mRNA levels measured across both fermentations showed the same characteristic changes during the course of the fermentation, demonstrating the reproducibility of gene expression measurements from biological duplicate experiments.

Clear differences were observed in the timing and magnitude of changes in mRNA abundance and their corresponding enzyme activities. This disparity should not be surprising. The level of gene expression in a cell is determined by several factors, including the rate of transcription initiation, the stability of the mRNA, the efficiency of translation, and post-translational events such as protein stability and modification (Carrier and Keasling 1997; Macdonald 2001; McCarthy and Gualerzi 1990; Mejia et al. 1992; Varshavsky 1996). However, few studies have been published showing experimental evidence for the existence of this disparity between transcriptome and proteome (Anderson and Seilhamer 1997; Gygi et al. 1999). For prokaryotes, nothing comparable has been reported to date.

Inferring enzymatic activity from transcriptome data is a complicated prospect. The simplest model would suggest a direct correlation between transcription and activity. From the accumulated data regarding the complexity of transcriptional and translational control, we would not expect most relationships to be so simple. Based on our results we would argue that the dynamic interplay of transcription and translation cannot be assessed, even qualitatively, without temporal data. Without monitoring expression over time, interpretation of the results could be misleading, and discrepancies in the timing or magnitude of transcription and translation would easily be missed.

A multitude of factors may account for discrepancies between measured gene expression and enzyme activity levels. It has been shown that one factor affecting gene expression is mRNA stability (Grunberg-Manago 1999). The stability of prokaryotic mRNAs can vary enormously between genes, and half-lives between 1 and 80 min have been observed (Alifano et al. 1994; Forchhammer et al. 1972; Pedersen and Reeh 1978). Furthermore, it has been found that the stability of specific transcripts can be altered within the cell over time in response to environmental changes, such as the growth rate (Nilsson et al. 1984). With this information, it seems conceivable that the initiation of a high-stability transcript could lead to an initial increase in the level of mRNA which would remain stable even if no further transcription was initiated. However, the translational machinery can translate a given mRNA many times into polypeptides which are then transformed into active enzyme. This would lead to a profile in which there is an initial increase in mRNA that is lagged by an enzyme activity, which then increases by a factor much greater than the increase in mRNA. One can also extend this scenario to include the situation in which degradation of the mRNA begins at a certain time point and, consequently, enzyme activity reaches a plateau.

Factors of mRNA stability and translational control can also be used to develop a model for circumstances in which transcript accumulation and enzyme activity appear non-correlated. A decision of whether to translate an mRNA may be applied non-specifically, through global effectors on the translation machinery, but alternatively individual mRNA features can impose mRNA-specific forms of control. These regulatory effects are thought to involve either direct blocking of the ribosomal binding area or the stabilization of an inhibitory mRNA secondary structure. Most examples of translational control are thought to affect the initiation of translation (Iost and Dreyfus 1995; McCormick et al. 1994), although there exists some experimental evidence that elongation rates can be regulated as well (Alifano et al. 1994; Macdonald 2001).

Echoing this complexity, our data suggest the existence of different underlying control mechanisms for expression of the tested genes. Relative levels of mRNA and enzyme activity, and changes therein, were examined over a stretch of almost 3 h. It seems conceivable that relatively high stability of *ilvA* mRNA could explain the delayed increase in threonine dehydratase enzyme activity relative to transcript accumulation. The profile observed for homoserine kinase (*thrB*) seems to be much different, but a similar model using mRNA stability and translational control can be applied to explain the differences in timing and magnitude. Our data for homoserine dehydrogenase come closest to fitting the canonical direct correlation between transcript accumulation and increase in enzyme activity. Even so, this interpretation of the data is merely an inference. It is important to keep in mind that similar profiles can be explained with many different models and many points of control. Differences in protein stability, post-translational modifications (although none are known for the studied proteins), and regulation of enzyme activity are other factors that complicate interpretation of the data. Presumably, different mechanisms of regulation of gene expression play a role here and could be the subject of detailed studies involving these genes. These data underscore the complexity of the problem of predicting enzyme activities from mRNA data and stress the need for incorporating external data in order to validate expression-profiling hypotheses.

The data presented here show that the global analysis of transcription with DNA microarrays has clear limitations in determining the impact of gene expression changes on cellular physiology. DNA microarray data are reliable and reproducible, as we confirmed by employing competitive RT-PCR as an independent method for measuring changes in gene expression. However, changes in mRNA abundance do not necessarily reflect changes in enzyme activity or intracellular concentrations of metabolites. Indeed, monitoring mRNA abundance, enzyme activities and metabolite production over time yielded insights into the complexity of the relationship between RNA levels, protein levels, and physiological changes. Few researchers have examined the correlation between transcription and changing in vivo

enzyme activities. Data from analysis of gene expression are often used to generate models of physiology (Ideker et al. 2001). As more studies examine the relationship between gene expression and protein levels in quantitative and biologically reproducible ways, it may be possible to identify genetic themes (e.g. RNA sequence or structural motifs) that can be used to predict translation efficiency and RNA stability. With integrated models based on the correlation of such bioinformatic and experimental data, it may someday be possible to predict phenotype directly from transcriptional information.

Deciphering the components that define a particular biological phenotype remains one of the greatest challenges in biology. DNA microarrays certainly will play a key role by revealing gene expression fingerprints that correlate with specific phenotypes, but only in combination with other emerging tools will we get closer to understanding the complex network of how genomes and their environments interact.

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