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Adaptation of *Corynebacterium glutamicum* to Nitrogen Starvation: A Global Analysis Using Transcriptome and Proteome Techniques

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Introduction

The regulation of nitrogen metabolism in the Actinomycetales was subject of research mainly in the last years (for review, see Burkovski, 2003a). This group of bacteria includes producers of antibiotics, e.g. *Streptomyces* species, pathogens such as *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*, as well as amino acid and nucleotide producers such as *Corynebacterium ammoniagenes*, *Corynebacterium efficiens*, and *Corynebacterium glutamicum*. For *C. glutamicum*, detailed information on the uptake and assimilation of different nitrogen sources is available. Moreover, the key components of nitrogen control were identified: AmtR, the master regulator of nitrogen control in *C. glutamicum*, GlnK, the sole P_{II}-type signal transduction protein in this organism, and two modifying enzymes, a putative adenylyltransferase and a putative uridylyltransferase (for review, see Burkovski, 2003b; 2004). This communication focuses on the investigation of the *C. glutamicum* nitrogen starvation response by transcriptome and proteome analysis. These global approaches were combined with the continuous cultivation of cells in a chemostat, which allows to establish highly defined growth conditions over a long period of time.

Transcriptome analyses

When *C. glutamicum* was challenged by nitrogen limitation, changes in the expression of more than 500 genes occurred. These include genes coding for transporters, proteins involved in nitrogen metabolism, protein synthesis, and regulation.

Transport proteins

The adaptation of the cellular transport capacity is a major response to nitrogen limitation. The expression of *amt* and *amtB* encoding ammonium transporters is highly increased and also urea, creatinine, and L-glutamate uptake is upregulated as indicated by the expression of the *urtABCDE* operon (G. Beckers, A. K. Bendt, R. Krämer, and A. Burkovski, submitted for publication), of the *crnT* gene (Bendt et al., 2004), and of the *gluABCD* cluster. Besides increased uptake of nitrogen sources, the transport of carbon

sources seems to be important for the cell since transcription of the *ptsG* and *ptsS* genes coding for proteins involved in sugar uptake as well as NCgl1968 and NCgl2463 coding for putative dicarboxylate transporters, is increased.

The expression of several other genes encoding membrane proteins is decreased in response to nitrogen limitation. These include *betP* and *putP* which code for compatible solute uptake systems. Compatible solutes are accumulated within the cell during hyperosmotic stress and do not serve as nitrogen or carbon source.

Nitrogen metabolism and amino acid biosynthesis

In response to nitrogen limitation, an enhanced transcription of the *gltBD* operon encoding glutamate synthase (GOGAT) and the *glnA* gene, encoding glutamine synthetase (GS) was observed. This promotes the assimilation of NH_4^+ via the high affinity GS/GOGAT pathway rather than by the low-affinity glutamate dehydrogenase (GDH). Additionally, an upregulation of the urease-encoding *ureABCEFGD* operon enables the utilization of urea, while the enhanced expression of *codA*, which codes for creatinine deaminase, is crucial for the degradation of creatinine to 1-methylhydantoin and ammonia (Bendt et al., 2004).

A down-regulation of genes encoding proteins for the synthesis of different L-amino acids was observed. Interestingly, expression of the *ddh* gene coding for meso-diamonopimelate dehydrogenase is downregulated, while transcription of *dapD* coding for tetrahydrodipicolinate-N-succinyltransferase is increased. These two enzymes are part of the split diaminopimelate pathway. Shut down of *ddh* expression impairs the low NH_4^+ affinity branch of this pathway, while upregulation of *dapD* transcription increases flux via the high affinity branch, allowing synthesis of the cell wall precursor diaminopimelate even under NH_4^+ limitation. To avoid drain of NH_4^+ towards lysine synthesis, which diverts from this pathway, expression of *lysA* (diaminopimelate decarboxylase gene) is repressed upon NH_4^+ limitation.

Carbon metabolism and energy generation

Another group of genes exhibiting increased transcription during nitrogen limitation are those encoding proteins involved in energy metabolism. Examples are *fda*, *gap*, and *acn*, which code for the fructose-1,6-diphosphate aldolase, glyceraldehyde dehydrogenase, and aconitase. Additionally, transcription of almost all genes encoding respiratory chain components was enhanced. The *aceA* and *aceB* genes, which code for isocitrate lyase and malate synthase, as well as *adhA*, coding for alcohol dehydrogenase, showed a downregulated transcription in response to ammonium limitation.

DNA replication, transcription, translation, protein synthesis and turnover

In response to nitrogen limitation, cells diminished their protein synthesis capacity. Transcription of genes encoding DNA polymerase I (*polA*), DNA-directed RNA polymerase α subunit (*rpoA*), various ribosomal proteins and translation initiation factors was decreased in nitrogen-starved cells, especially in shaking flask experiments. The increased transcription of the *groEL* and *groES* genes, which code for the GroEL chaperonine and the GroES co-chaperonine indicate a tendency towards protein stabilization in

nitrogen-starved cells.

Cell division and cell wall synthesis

Since nitrogen limitation impairs growth, the cell division machinery of *C. glutamicum* might be at least partially dispensable under these conditions. In fact, transcription of *ftsI* encoding the cell division protein FtsI, NCgl2081 coding for a putative UDP-N-acetylmuramyl pentapeptide phosphotransferase, and NCgl1366, coding for a putative ATPase involved in chromosome partitioning was decreased.

Influence on global regulatory systems

Our transcriptome analyses showed that *glnD* and *glnK* encoding central components in nitrogen signal transduction are expressed during nitrogen limitation. This is crucial for the cellular response to nitrogen starvation (Nolden et al., 2001). However, metabolic pathways in a cell do not work isolated but are connected by common metabolites and global regulatory systems. This regulation network and intersystem cross-talk is indicated by the altered expression of genes encoding various transcriptional regulators and two different two-component signal transduction systems.

Growth rate-depending effects on transcription

The chemostat experiments allowed not only insights into nitrogen-dependent expression of genes, but also growth rate-dependent transcription. When different chemostat experiments were compared, genes were identified, which showed a growth-rate dependent expression pattern. Transcription of genes encoding ATP synthase subunits b, δ, α, γ β, and ϵ, of the *tkt* gene coding for transketolase, the *galU2* gene for UDP-glucose pyrophosphatase, the *pgi* gene for glucose-6-phosphate isomerase, and of NCgl2579 encoding carbonic anhydratase was increased in fast growing cells. In contrast, transcription of the *mez* gene, coding for malic enzyme, and sigma factor encoding *sigB* and *sigE* genes is decreased in slow growing cells. Total growth arrest in shaking flask experiments with nitrogen-free medium resulted additionally in the decreased transcription of 38 genes coding for ribosomal proteins.

Proteome analysis

Previously carried out proteome analyses by two-dimensional gel electrophoresis (for review, see Hermann et al., 2001; Schaffer & Burkovski, 2004) revealed that differences in the protein profile of cells grown under nitrogen-rich conditions and nitrogen-starved cells are hardly detectable when conventional staining techniques (i. e. Coomassie or silver-staining) were used. Therefore, in different studies *in vivo* [^{35}S]methionine labeling and autoradiography has been used to improve the sensitivity of proteome analysis (Schmid et al., 2000; Nolden et al., 2001). The use of continuous fermentation is an interesting alternative to *in vivo* labeling techniques, since the cells can adjust to a constant environment for a long period of time, which significantly improves the sensitivity of the approach.

Spots with different intensity and size depending on the nitrogen supply were excised from 2-D gels and

the proteins were identified by peptide mass fingerprint (Fig. 1). Protein spots with increased size on gels loaded with cell extract from nitrogen-deprived cultures were identified as urease subunits (*ureB*, *ureC*, *ureG*, *ureD*), a putative ornithine cyclodeaminase (*ocd*), creatinine deaminase (*codA*), the GlnK protein, tetrahydrodipicolinate-N-succinyltransferase (*dapD*), and glutamine synthetase (*glnA*). Besides these proteins involved in nitrogen metabolism, proteins of carbon metabolism were found, namely, glyceraldehyde-3-phosphate dehydrogenase (*gap*), aconitase (*acn*), and the ATP synthetase F_1 δ subunit (*atpH*). Additionally, the GroEL chaperone protein, a putative L-2,3-butanediol dehydrogenase (*butA*), 5-enolpyruvylshikimate-3-phosphate synthase (*aroA*), phosphorglycerate dehydrogenase (*serA*), glycine hydroxymethyltransferase (*glyA*), a thiamine pyrophosphate-requiring enzyme (NCgl2521), and two hypothetical proteins (NCgl2450, NCgl2451) were found to be present in higher amounts during nitrogen limitation.

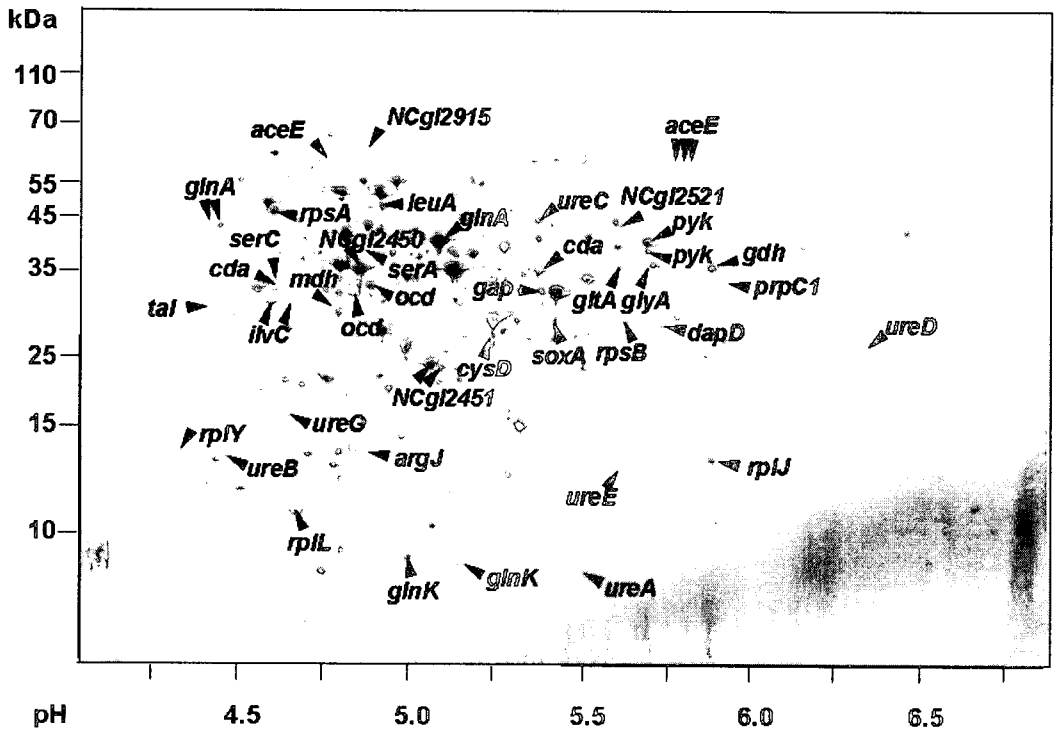

Fig. 1. Comparison of *C. glutamicum* protein profiles from cells grown with different nitrogen supply. Cytoplasmic protein fractions were separated by 2-D PAGE and stained with Coomassie-brilliant blue. For comparison, a false color overlay was produced using the DECODON Delta2D 3.1 software (red: nitrogen-rich batch phase, green: nitrogen-limited continuous phase, spot designation according to annotation of the corresponding genes).

Proteins with decreased concentration in response to nitrogen starvation were isopropylmalate synthase (*leuA*), ketol acid reductoisomerase (*ilvC*), glutamate N-acetyltransferase (*argJ*), and sulfate adenylate transferase subunit 2 (*cysD*), which are involved in the synthesis of different amino acids, as well as peptidyl-prolyl cis-trans isomerase (*ppiA* gene product, rotamase).

Also protein modifications can be detected by proteome analyses. We were able to identify the

modification of GlnK, which is an AMP group. Additionally, a pI shift of glutamine synthetase from approximately 4.6 to 5.1 was observed when cells were grown under nitrogen limitation. This is in accord with a deadenylylation of the GS enzyme in response to nitrogen deprivation, which was reported by us previously.

Discussion

The response of *C. glutamicum* to nitrogen starvation was studied by transcriptome and proteome analyses in combination with two different cultivation techniques, growth in shaking flasks and nitrogen-limited chemostat bioreactors. A major advantage of the cultivation in a continuous fermentation mode is the possibility to establish highly defined long-term conditions of nutrient limitation, while in shaking flasks only a total lack of nutrients can be guaranteed for a long time period. Using this combination of cultivation methods we were able to distinguish between specific and general cellular responses to the different nutrient limitations and to separate these from growth rate depending regulatory mechanisms.

Scavenging of nutrients and rearrangement of metabolic pathways is a major response to nitrogen starvation in *C. glutamicum*. Both nitrogen and carbon metabolism are affected, since *C. glutamicum* has an extremely high energy demand in case of a complete fixation of ammonium via the GS/GOGAT pathway (Schmid et al., 2000). Therefore, the necessity of an increased ATP production during nitrogen starvation becomes obvious and, as a consequence, increasing synthesis of glycolytic enzymes and redox chain components is a reasonable response to nitrogen starvation. A prerequisite for these changes in the energy metabolism is a cross-talk between the different global regulatory systems of nitrogen control and carbon metabolism, which will be investigated in future studies.

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