Response to nitrogen starvation in *Corynebacterium glutamicum*

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Abstract

Proteins strongly synthesized in *Corynebacterium glutamicum* during nitrogen restriction were examined by two-dimensional gel electrophoresis and microsequencing. Two main groups of enzymes were identified beside miscellaneous proteins, enzymes involved (i) in protein synthesis, and (ii) in carbon metabolism. Biochemical measurements revealed an increase of oxygen consumption during nitrogen starvation, indicating an enhanced energy demand of the cells. By Northern hybridizations, an increased transcription for the gap and fda genes upon nitrogen deprivation was shown. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

*Corynebacterium glutamicum* is a Gram-positive soil bacterium which is applied in fermentation processes on industrial scale. By the use of different mutant strains, not only large amounts of glutamate (800 000 t/a) but also lysine (250 000 t/a) are produced, beside smaller amounts of alanine, isoleucine and proline [8]. While during industrial amino acid production the supply of nutrients is in general sufficient, in its natural environment, the soil, *C. glutamicum* has to cope with a variety of nutrient restrictions. One of the major limitations is the abundance of nitrogen sources. In *C. glutamicum*, the GS/GOGAT system is induced upon nitrogen starvation [17] and a (methyl)ammonium uptake carrier [15] as well as a urea permease [16] is synthesized. However, nothing is known about the global response of *C. glutamicum* to nitrogen starvation until now. Therefore, we started an approach to identify proteins synthesized during nitrogen deprivation using two-dimensional (2-D) gel electrophoresis and microsequencing. In addition, key energetic parameters of the cells were measured and Northern hybridizations were carried out.

2. Materials and methods

2.1. Bacterial strains and growth

*C. glutamicum* wild-type strain ATCC 13032 [1] was grown at 30°C. For nitrogen starvation experiments, cells were grown for 8–10 h in BHI medium (Difco, Detroit, MI, USA), transferred to minimal medium [16] for overnight growth and then used to inoculate fresh minimal medium to an OD 600 of approximately 0.5. When the exponential growth phase (OD 600 3–4) was reached, the culture was harvested, washed and resuspended in prewarmed minimal medium without nitrogen source. Phosphate starvation was induced by incubation in phosphate-free medium.

2.2. Labeling with [35S]methionine, 2-D gel electrophoresis and microsequencing

Cells were grown and resuspended in prewarmed minimal medium without nitrogen source as described. To 20-ml aliquots with an OD 600 of about 5, 2 µl of [35S]methionine solution (specific activity 370 mBq mJ⁻¹; Hartmann Analytic, Braunschweig, Germany) was added. After 30 min, 0.1 mM methionine (final concentration) was added and cells were harvested after 10 min of incubation. Preparation of cytoplasmic and membrane-associated
ated proteins, 2-D polyacrylamide gel electrophoresis (PAGE) and microsequencing were carried out as described [6]. Gels were loaded with 60 000 cpm, after 2-D PAGE, [35S]methionine-labeled proteins were detected using BAS-MP 2025 imaging plates and a Bio-Imaging Analyzer BAS-1800 (Fuji, Omiya, Japan).

2.3. Determination of oxygen consumption and intracellular ATP, ADP and AMP pools

A Clark electrode (Oxygraph, Hansatech Instruments, Kings Lynn, UK) was used to measure the oxygen consumption of cells. For this purpose, the cell suspension was transferred from the shaking flask to the electrode prewarmed to 30°C and incubated under stirring. Internal ATP concentrations were determined as described [5] using the firefly luciferin/luciferase assay, AMP and ADP concentrations were determined via reversed-phase chromatography using a Lichrosorb RP-18 column (solution A: 10 mM tetrabutylammonium hydrogensulfate, 15 mM potassium phosphate, pH 5.0; solution B: 10 mM tetrabutylammonium hydrogensulfate, 35 mM potassium phosphate, pH 5.0, 50% acetonitrile; gradient: 1^99% B).

2.4. Molecular biology techniques

Cloning of DNA and subsequent transformation steps were carried out using standard techniques [13]. Total RNA was prepared after disruption of the cells by glass beads using the RNeasy Mini kit (Qiagen, Hilden, Germany). The RNA was size-fractionated using agarose gels containing formaldehyde [13] and blotted onto nylon membranes (Boehringer Mannheim, Germany). For hybridization, a digoxigenin-labeled 0.5-kb gap [3] fragment, a 0.5-kb fda [19] probe and a 0.5-kb fragment of C. glutamicum 16S rRNA gene [12] were prepared by PCR using digoxigenin-labeled dNTPs (PCR DIG Labeling Mix Plus; Boehringer Mannheim, Germany) and CSPD as light-emitting substrate.

3. Results and discussion

3.1. Proteins synthesized during nitrogen starvation

In previous studies, we investigated several proteins involved in the response of C. glutamicum to nitrogen deprivation, e.g. ammonium and urea uptake systems [15,16], or GS [7]. In order to identify until now unrecognized proteins synthesized during these conditions, we started a more global approach. Cells were grown in minimal medium and subsequently starved for nitrogen in the presence of glucose. Proteins newly synthesized under these conditions were labeled by [35S]methionine, the corresponding spots were excised from Coomassie brilliant blue-stained gels, and the proteins were analyzed by microsequencing.

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>N-terminal sequence</th>
<th>Homologous protein</th>
<th>Identity (%)</th>
<th>Organism</th>
<th>Accession no. (SwissProt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>XXVAMYTGGGAGIRKG1</td>
<td>putative l-2,3-butanediol dehydrogenase</td>
<td>81</td>
<td>Mycobacterium leprae</td>
<td>Q50066</td>
</tr>
<tr>
<td>N2</td>
<td>ANYTAADVKKRLTLAGMDCKK</td>
<td>(putative) elongation factor TS</td>
<td>96</td>
<td>(C. glutamicum), Streptomyces coelicolor</td>
<td>(P42439), O31213</td>
</tr>
<tr>
<td>N3</td>
<td>GNVNNYITETIHTPLVLKLN</td>
<td>cysteine synthase</td>
<td>72</td>
<td>B. subtilis</td>
<td>P37887</td>
</tr>
<tr>
<td>N4</td>
<td>AVKLKDLDEG</td>
<td>phosphoglycerate kinase</td>
<td>100</td>
<td>C. glutamicum</td>
<td>Q01655</td>
</tr>
<tr>
<td>N5</td>
<td>AEKVHHVIAEILD</td>
<td>enolase</td>
<td>75</td>
<td>Pyrococcus furiosus</td>
<td>G884993</td>
</tr>
<tr>
<td>N6</td>
<td>AGIMHFFARGILD</td>
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<td></td>
<td></td>
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<tr>
<td>N9</td>
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<td>fructose-bisphosphate aldolase</td>
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</tr>
<tr>
<td>N11</td>
<td>LKNPEXPFXNLAL</td>
<td>phosphoglycerate kinase (internal fragment)</td>
<td>58</td>
<td>Borrelia burgdorferi</td>
<td>Q59181</td>
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<tr>
<td>N12</td>
<td>TIRVGINGFGRGNNNF</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>N21</td>
<td>AKTHYQGNETATGSELLQVG</td>
<td>putative thiol peroxidase</td>
<td>47</td>
<td>Clostridium pasteurianum</td>
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<tr>
<td>N6</td>
<td>AEIMHVFAREILDSGN</td>
<td>enolase</td>
<td>75</td>
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<tr>
<td>N62</td>
<td>AEILTHSSDEIRASIAN</td>
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<td>M. leprae</td>
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<tr>
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<td>M. tuberculosis</td>
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<td>cysteine synthase</td>
<td>80</td>
<td>Streptococcus gordonii</td>
<td>Cab40550</td>
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<tr>
<td>N150</td>
<td>ANPLSAAKYL</td>
<td>unknown</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The single letter code is used to indicate the different amino acids (X, amino acid unknown). For spots no. N7, N8, N10, N13-N19, NM3, NM4, NM6, NM7, NM10 and NM12, no sequences were determined due to blocked N-termini or insufficient amounts of protein.
blue-stained gels run in parallel, blotted onto PVDF membranes, and the N-terminal amino acid sequences were determined.

From 21 spots of the cytoplasmic protein fraction analyzed (Fig. 1A), nine were identified while two were unknown and for 10, no sequence was determined due to insufficient amounts of protein or blocked N-termini. The proteins identified by sequence similarity can be divided into different groups depending on their function: most were enzymes of the central carbon metabolism, namely phosphoglycerate kinase (N4, N11), enolase (N5), fructose-1,6-bisphosphate aldolase (N9) and glyceraldehyde-3-phosphate dehydrogenase (N12). Additionally, proteins involved in protein synthesis, namely elongation factor EF-Ts (N2) and cysteine synthase (N3), were found. The appearance of a 1,2,3-butanediol dehydrogenase (N1) and a putative thiol peroxidase (N21) is unclear.

From the 15 proteins of the membrane fraction (Fig. 1B), four proteins were identified, five were unknown and from six, no sequence could be determined. The K subunit of ATP synthase (NM2) is part of the ATP generating enzyme. Two other proteins, enolase (NM1) and CysK (NM14), are water-soluble proteins, also identified in the cytoplasmic fraction. Both proteins were previously found in the C. glutamicum membrane fraction [6], most likely due to their high abundance in the cell. The role of the 35-kDa protein (NM11) is unclear.

The fact that no proteins were identified, which were

Fig. 1. 2-D pattern of (A) cytoplasmic proteins and (B) proteins of the membrane fraction labeled with [35S]methionine (first dimension pH 4-7; second dimension 8-18% sodium dodecyl sulfate gradient gel); proteins excised for microsequencing are numbered and listed in Table 1. The apparent molecular masses of marker proteins in kDa are indicated.
already known to be induced upon nitrogen deprivation, might be the result of a low expression rate of the corresponding genes. For example, the (methyl)ammonium uptake system Amt is fully induced only after 2–3 h [15], the urea permease only after 3–4 h [16]. Time-resolved data for the synthesis of other proteins like GS, GOGAT and urease are missing. Additionally, enzymes which are subject to post-transcriptional modification depending on the nitrogen status of the cell like GS [7] might be first upregulated on the level of activity before more protein is synthesized.

3.2. Effect of nitrogen starvation on energetics of the cell

The results obtained by proteome analysis indicated an effect of nitrogen deprivation on the bioenergetics of C. glutamicum. As key energetic parameters, oxygen consumption and intracellular ATP concentration were determined. While the ATP pool stayed constant (3.1 ± 0.1 mM before versus 2.9 ± 0.3 mM after deprivation of nitrogen), oxygen consumption increased depending on the time of nitrogen starvation from approximately 8 nmol ml⁻¹ min⁻¹ to more than 13 nmol ml⁻¹ min⁻¹ (Fig. 2A), indicating a rising energy consumption. Respiration of control cells did only increase slightly (Fig. 2B). In contrast to the nitrogen-deprived culture, in this case, however, also the cell number increased. To exclude a possible effect of nitrogen deprivation on adenine nucleotide pools, beside ATP also ADP and AMP concentrations were measured. No significant changes in the ATP/ADP ratio or the total amount of adenosine nucleotides were observed (data not shown).

For Escherichia coli, it was shown that about 18 500 µmol of ATP is required for the synthesis of 1 g dry mass when glutamate is provided by GDH while GS is inactive in the presence of sufficient amounts of ammonia [11]. If, however, ammonia fixation is catalyzed by GS, the ATP demand increases by about 30% to a total of about 24 850 µmol (g dry weight)⁻¹. For C. glutamicum, the demand for ATP in case of a complete fixation of ammonium via GDH in contrast to assimilation exclusively via GS is even higher. About 6350 µmol of amino groups per g dry mass is transferred from glutamate to amino groups of biomass building blocks via transaminase reactions [9,10], approximately the double amount as necessary in E. coli. Although these calculations are based on the nitrogen-assimilating enzymes only, the physiological necessity of an enhanced ATP production during nitrogen starvation becomes obvious and, as a consequence, increasing the amount of glycolytic enzymes and ATP synthase is a reasonable response in terms of physiology to nitrogen starvation.

It might be argued that the observed effects on the bioenergetics of C. glutamicum are not specific for nitrogen restriction, but reflect a general starvation response of the cells. Therefore, we examined also the response of the C. glutamicum wild-type to phosphate deprivation, which is a well characterized, e.g. in Bacillus subtilis [4] and E. coli [18]. Compared to control cells, no increase of oxygen consumption was observed in this case (data not shown), indicating that enhanced oxygen consumption is not a general starvation effect. During hyperosmotic stress, however, oxygen consumption of C. glutamicum cells increases, too (H. Rönsch, personal communication). Since the general stress response of C. glutamicum has not been investigated until now, we cannot exclude that nitrogen starvation interferes with the general stress response of the cell. This was observed, e.g., in B. subtilis, where synthesis of several stress proteins is induced upon glucose starvation [2].
3.3. Transcription of genes encoding glycolysis enzymes

The data obtained by 2-D PAGE and by the measurement of oxygen consumption indicated an increased energy metabolism of the cells upon nitrogen starvation. In principle, this should also be detectable on the level of transcription. Therefore, we performed Northern hybridization experiments probing genes encoding glycolysis enzymes. As an example, transcription of the \textit{gap} gene, encoding glyceraldehyde-3-phosphate dehydrogenase, was investigated. It was assumed previously that this gene is constitutively expressed, changes in mRNA levels were not observed \cite{3}. Total RNA was extracted from cells 5 min before and after the begin of nitrogen starvation. Equal amounts of the different RNA preparations were size-fractionated by agarose gel electrophoresis. (A) Transcription of the \textit{gap} gene 5 min before (1) and 5 min after nitrogen deprivation (2); (3) RNA standard (from top to bottom 6.9, 4.7, 2.7, 1.8, 1.5, 1.0, 0.6, 0.4 and 0.3 kb). (B) Transcription of the \textit{fda} gene 5 min before (1), and 5, 15 and 30 min (2–4) of nitrogen starvation. (C) 16S rRNA probed for control (for explanation, see A).

3.4. Concluding remarks

The data obtained in this study indicate a cross-talk between nitrogen starvation response and carbon metabolism in \textit{C. glutamicum}. Such a connection would be advantageous to coordinate the central metabolism of the cell and to provide sufficient energy for the active uptake of ammonium and its fixation via glutamine synthetase. Furthermore, the results revealed that nitrogen regulation intersects with energy metabolism in an unexpected way and it is an advantage of 2-D gel electrophoresis and microsequencing to reveal such responses that otherwise might have been overlooked.

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References


