Changes in protein synthesis during the adaptation of *Bacillus subtilis* to anaerobic growth conditions

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After a shift of *Bacillus subtilis* from aerobic to anaerobic growth conditions, nitrate ammonification and various fermentative processes replace oxygen-dependent respiration. Cell-free extracts prepared from wild-type *B. subtilis* and from mutants of the regulatory loci fnr and resDE grown under aerobic and various anaerobic conditions were compared by two-dimensional gel electrophoresis. Proteins involved in the adaptation process were identified by their N-terminal sequence. Induction of cytoplasmic lactate dehydrogenase (LctE) synthesis under anaerobic fermentative conditions was dependent on fnr and resDE. Anaerobic nitrate repression of LctE formation required fnr-mediated expression of narGHJI, encoding respiratory nitrate reductase. Anaerobic induction of the flavohaemoglobin Hmp required resDE and nitrite. The general anaerobic induction of *ywfI*, encoding a protein of unknown function, was modulated by resDE and fnr. The *ywfI* gene shares its upstream region with the *pta* gene, encoding the fermentative enzyme acetyl-CoA:orthophosphate acetyltransferase. Anaerobic repression of the synthesis of a potential membrane-associated NADH dehydrogenase (*yjID*, Ndh), and anaerobic induction of fructose-1,6-bisphosphate aldolase (*fbaA*) and dehydrodiposphamide dehydrogenase (*phdD*, *lpd*) formation, did not require fnr or resDE participation. Synthesis of glycerol kinase (*glpK*) was decreased under anaerobic conditions. Finally, the effect of anaerobic stress induced by the immediate shift from aerobic to strictly anaerobic conditions was analysed. The induction of various systems for the utilization of alternative carbon sources such as inositol (*iolA*, *iolG*, *iolH*, *iolI*), melibiose (*melA*) and 6-phospho-α-glucosides (*glvA*) indicated a catabolite-response-like stress reaction.

**Keywords:** anaerobic growth, *Bacillus subtilis*, two-dimensional gel electrophoresis, stress response

INTRODUCTION

The natural habitat of *Bacillus subtilis*, the upper layers of soil, is characterized by changes in oxygen tension. Recently, the anaerobic energy metabolism of *B. subtilis* employing nitrate ammonification and various fermentation pathways was discovered (Cruz-Ramos *et al.*, 1995; Hoffmann *et al.*, 1995, 1998; Nakano *et al.*, 1997; Nakano & Zuber, 1998). *B. subtilis* reduces nitrate to nitrite using the respiratory nitrate reductase encoded by *narGHI* (Cruz-Ramos *et al.*, 1995; Hoffmann *et al.*, 1995). Transcription of the *narGHIJ* operon is induced under anaerobic conditions by the redox regulator Fnr and the multifunctional two-component regulatory system encoded by *resDE*. During this process, *fnr* transcription is under the control of *resDE* (Cruz-Ramos *et al.*, 1995; Nakano *et al.*, 1996). Nitrite is further reduced to ammonia by a soluble NADH-dependent nitrite reductase NasDE (Hoffmann *et al.*, 1998; Nakano *et al.*, 1998). This dissimilatory activity of the enzyme significantly enhances anaerobic growth via NAD\(^+\) regeneration (electron sink). Under aerobic conditions...
the same nitrate reductase is part of the assimilatory pathway for the incorporation of nitrogen into cellular building blocks such as amino acids and nucleotides. This dual function is reflected by the two levels of regulation, with the involvement of the nitrogen regulator TnRA under aerobic conditions and ResDE under anaerobic conditions (Nakano et al., 1998).

Fermentation by B. subtilis results in the formation of lactate, acetate, 2,3-butanediol, succinate and ethanol (Nakano et al., 1997). Less is known about the regulation of the various fermentative processes and their coordination with anaerobic respiration. Here we present an experimental approach using various gel electrophoretic techniques combined with N-terminal amino acid sequence determination for the identification of proteins involved in the adaptation process to anaerobic growth conditions in B. subtilis.

METHODS

Strains, growth conditions and extract preparation. Growth of B. subtilis strains JH642 (trpC2 pheA1, BGSC 1A96), THB2 (JH642, fnr) and LAB2313 (JH642, resDE Pspac-fnr) was as described by Hoffmann et al. (1998) and Nakano et al. (1998). Anaerobic stress conditions were achieved as outlined before (Hoffmann et al., 1995). The cytoplasmic and membrane fractions of cell-free extracts were separated as described by Hoffmann et al. (1998) and Nakano et al. (1998). The successful separation of the cytoplasmic and membrane fractions was verified by determination of soluble porphobilinogen synthase, glutamyl-tRNA reductase and membrane-bound respiratory nitrate reductase activities (Hoffmann et al., 1998). No cross-contamination was observed.

Two-dimensional gel electrophoresis of soluble proteins. The soluble fractions of aerobically and anaerobically grown B. subtilis strains were separated by two-dimensional gel electrophoresis as described by Moebius et al. (1997) and Rompf et al. (1998). Proteins observed after Coomassie blue staining were quantified using the ImageMaster UDS system (Pharmacia), following the instructions of the manufacturer. Major protein spots which did not change in their intensities under the various tested growth conditions served as standards for the protein amounts employed.

Blue native gel electrophoresis of membrane-associated proteins. B. subtilis cells were grown anaerobically and aerobically in supplemented Luria–Bertani medium containing 10 mM NaNO₃ as described before (Hoffmann et al., 1998). Cells were harvested, washed in 67 mM KPO₄ buffer pH 7-4 including 0.87% KCl, resuspended in the same buffer supplemented with lysozyme (0.2%, w/v) and DNase I (0.2% w/v), stirred at room temperature for about 1 h, and then centrifuged at 3500 g for 10 min to remove cell debris. The membranes were recovered from the supernatant by a high-speed (50000 g) centrifugation. The resulting pellet was washed four times with water, resuspended in 10 mM Tris pH 8.0 including 4% CHAPS, and solubilized overnight by gentle stirring on ice. The suspension was centrifuged at 100000 g for 1 h. The supernatant contained the solubilized membrane and membrane-associated proteins. Proteins in 50 µL samples (~250 µg protein) of the supernatant were separated by blue native gel electrophoresis in the first dimension and SDS gel electrophoresis in the second dimension (Schägger & von Jagow, 1991).

N-terminal protein sequence determination of identified proteins. The Coomassie-stained protein spots were cut from the gels and transferred onto a PVDF membrane (Millipore) by electroblotting. The proteins were sequenced on an Applied Biosystems A473a protein sequencer as described previously (Schmid et al., 1997; Völker et al., 1994). Each spot gave a single amino acid sequence, indicating the purity of the preparation. A search for amino acid sequence homologies of the obtained sequences with other proteins was carried out by screening the SubtiList database and the non-redundant database at NCBI (which includes GenBank, SWISS-PROT and PIR) with the BLAST program (BLAST 62 matrix) (Altschul et al., 1990; Moszet et al., 1995).

Enzyme assays. Lactate dehydrogenase, nitrite reductase, glycerol kinase and fructose-1,6-bisphosphate dehydrogenase
activity assays were performed as described before (Hayashi & Lin, 1967; Hoffmann et al., 1998; Nakano et al., 1998; Tarmy & Kaplan, 1968; Ujita & Kimura, 1982).

RESULTS AND DISCUSSION

Differences in protein production due to changes in oxygen tension

Aerobically pre-grown *B. subtilis* cultures were further incubated anaerobically with the addition of nitrate or nitrite, or without further additions in the medium. Residual oxygen from the inoculum was consumed by the cultures in less than 1 h, resulting in strictly anaerobic conditions (Hoffmann et al., 1998). This transition period with continuously decreasing oxygen tension was essential for the induction of the appropriate oxygen-independent enzymic systems and ensured continuation of growth. To analyse the cellular responses of *B. subtilis* to these various anaerobic growth conditions, protein extracts were prepared from the cytoplasmic and membrane cellular fractions and compared via two-dimensional gel electrophoresis. Differences in the observed protein patterns after Coomassie blue staining were further investigated by N-terminal sequence determination.

In both cytoplasmic and membrane fractions the concentration of 11 proteins varied significantly over ten independent experiments in dependence on differences in oxygen tension and the presence or absence of alternative electron acceptors (Fig. 1). Only protein spots which varied reproducibly in their cellular concentration due to changes of the growth conditions were subjected to N-terminal amino acid sequence determination. Four proteins were found to be presumably blocked at their N-terminus. The lack of sequence data did not result from insufficient protein recovery from the

<table>
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<th>Protein</th>
<th>Function</th>
<th>N-terminal sequence</th>
<th>Accession no.</th>
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<tr>
<td>GlpK</td>
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<td>METTYLILSLDQGTTSDRAILFNKEG</td>
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<td>YwfI</td>
<td>Unknown</td>
<td>SEQQMTNEAAKT</td>
<td>P39645</td>
</tr>
<tr>
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<td>Unknown; similar to NADH dehydrogenase</td>
<td>SKHIVILGAG</td>
<td>–</td>
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<tr>
<td>FbaA</td>
<td>Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13)</td>
<td>PLVSMTMLNTAK</td>
<td>P13243</td>
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<td>PhdD (Lpd)</td>
<td>Dihydrolipoamide dehydrogenase, E3 subunit of pyruvate dehydrogenase/2-oxoglutarate dehydrogenase (EC 1.8.1.4)</td>
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<td>P21880</td>
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<td>MelA</td>
<td>α-β-Galactoside galactohydrolase (EC 3.2.1.22)</td>
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<td>–</td>
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<tr>
<td>IolH</td>
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<td>MKLALDPSMYRDLTTLE热闹EMVVKTAE</td>
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<tr>
<td>IolG</td>
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<tr>
<td>GlvA</td>
<td>6-Phospho-α-glucosidase (EC 3.2.1.122)</td>
<td>M K K SFLIVIAGXGXTF</td>
<td>P54716</td>
</tr>
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Aerobic Anaerobic

Fig. 2. Analysis of membrane proteins and membrane-associated proteins extracted from B. subtilis grown under aerobic and anaerobic conditions in the presence of 10 mM nitrate using blue native gel electrophoresis in the first dimension and SDS gel electrophoresis in the second dimension. N-terminal amino acid sequences of the indicated proteins were determined.

gels since the purity and amount of protein on the membrane subjected to Edman degradation was determined for each experiment. Additional experiments including protease treatment of the proteins prior to N-terminal sequence determination or, alternatively, the determination of their molecular masses using mass spectroscopy will be the subject of future investigations.

The current experiments found that lactate dehydrogenase (LctE), flavohaemoglobin (Hmp), dehydrodipamid dehydrogenase (PhdD, Lpd), fructose-1,6-bisphosphate aldolase (FbaA) and the protein YwfI of unknown function were induced under anaerobic conditions (Table 1). A protein with similarity to NADH dehydrogenase (YjlD, Ndh) and glycerol kinase (GlpK) were found to be exclusively produced under aerobic growth conditions. The observed changes in protein synthesis varied under anaerobic conditions in dependence on the presence of nitrate and nitrite (Figs 2 and 3). No obvious effect of nitrate or nitrite on the protein patterns was observed for extracts prepared from aerobically grown B. subtilis.

The observed marked anaerobic induction of LctE indicated a central role of lactate dehydrogenase for the regeneration of NAD⁺ during fermentative growth. In the presence of the energetically more efficient nitrate respiration, the activity of the less efficient fermentation process was decreased and, accordingly, lactate dehydrogenase expression was significantly repressed. The involvement of the previously identified B. subtilis redox regulatory systems encoded by resDE and fnr in the observed anaerobic induction and nitrate repression of LctE formation was investigated. For this purpose, two-dimensional gels of extracts prepared from wild-type cells were compared to extracts prepared from resDE and fnr mutants. The amounts of LctE were quantified using an automated scanning and integration system. The highest amount of LctE observed in the wild-type, found under anaerobic fermentative conditions, was set to 100% and all other values were related to it (Fig. 4a).

In the resDE mutant, overall anaerobic LctE induction was significantly reduced compared to the wild-type, but nitrate repression was still visible. Mutation of fnr reduced the anaerobic LctE formation to approximately 50% and almost completely abolished the nitrate repression. The 5’ region of lctE contains two potential Fnr-binding sites, indicating the participation of Fnr in lctE expression. The fnr gene is also essential for nitrate synthesis.
Adaptation of *B. subtilis* to anaerobic conditions

**Fig. 3.** Two-dimensional gel electrophoresis of cytoplasmic proteins extracted from *B. subtilis* grown under anaerobic stress. *B. subtilis* was grown under strictly aerobic conditions and shifted to strictly anaerobic conditions in the presence of 10 mM nitrate. N-terminal amino acid sequences of the indicated proteins were determined.

**Fig. 4.** Synthesis of (a) LctE and (b) Hmp in dependence on oxygen tension, the presence of alternative electron acceptors and mutations in regulatory genes. The cytoplasmic fraction of cell-free extracts prepared from *B. subtilis* JH642 (wild-type), THB2 (*fnr*), LAB2313 (*resDE*) and THB1 (*narGH*) was separated by two-dimensional gel electrophoresis and proteins of interest were quantified as outlined in Methods (Hoffmann *et al.*, 1998; Nakano *et al.*, 1998). The error bars indicate SD (n = 3).

reductase formation (Cruz-Ramos *et al.*, 1995). The observation that the nitrate repression of LctE formation was completely abolished in the *fnr* mutant prompted us to investigate the effect of a *narGH* mutant on the observed nitrate repression. As shown in Fig. 4(a), almost equal anaerobic LctE formation was observed in the presence and absence of nitrate. These results suggested an indirect role of *fnr* via *narGHJI* induction in the observed nitrate-dependent regulation of LctE synthesis. The exact nature of the *B. subtilis* nitrate-regulatory system involving nitrate reductase activity remains to be determined.
**Regulation of Hmp formation by oxygen tension and nitrite**

Initially, under anaerobic conditions in the presence of nitrate the flavohaemoglobin Hmp was found to be strongly induced (Figs 1 and 4b, Table 1). Reinvestigation using nitrite as alternative electron acceptor and, as negative control, nitrate in combination with a *B. subtilis* nitrate reductase mutant identified nitrite as the second inducer for Hmp formation besides anaerobic conditions (Fig. 4b). These findings are in good agreement with genetic data for *B. subtilis* hmp regulation described by LaCelle et al. (1996). Those authors described the isolation of the hmp gene via random insertion of promoterless reporter genes into the *B. subtilis* chromosome and subsequent screening for anaerobic reporter gene induction. They showed that anaerobic transcription of hmp is dependent on resDE.

Again, in agreement with their regulatory studies significant reduction of Hmp formation in a resDE mutant under all anaerobic conditions tested was observed by two-dimensional gel electrophoresis (Fig. 4b).

However, the function of Hmp in anaerobic metabolism is still unknown. No obvious phenotype of a hmp mutant under various anaerobic growth conditions tested was observed (LaCelle et al., 1996). Previously, due to the nitrite regulation of hmp and structural features of the protein, an involvement of *B. subtilis* Hmp in nitrite reductase activity was suggested (LaCelle et al., 1996). To investigate the participation of hmp in nitrite reduction, the NADH-dependent nitrite reductase activities of wild-type cells, a hmp mutant and a hmp nasD double mutant were compared. No obvious changes in nitrite reductase activity or nitrite to ammonia conversion due to the presence or absence of intact hmp were observed (data not shown), making the participation of hmp in nitrite reduction very unlikely. Similar experiments investigating the participation of hmp in nitrate reduction also excluded hmp involvement in the initial step of nitrate ammonification (data not shown). The physiological function of Hmp in *B. subtilis* remains to be elucidated.

**YwfI formation is induced under anaerobic conditions**

A protein generally induced under all anaerobic conditions tested, encoded by the open reading frame ywfI, was identified (Figs 1 and 5a, Table 1). Database searches...
revealed significant homology between the deduced YwfI and proteins of unknown function from *Streptomyces coelicolor* (AL023517), *Mycobacterium tuberculosis* (Z80225) and *Mycobacterium leprae* (U15181).

Mutation of *fnr* significantly reduced anaerobic YwfI induction only under anaerobic fermentative conditions (Fig. 5a). Since no obvious potential Fnr-binding site was found in the 5′ region of *ywfI* the influence of *fnr* on YwfI formation might be indirect. Mutation of *resDE* increased YwfI synthesis under anaerobic fermentative conditions and in the presence of nitrite (Fig. 5a). The molecular basis of this observation remains to be determined. Interestingly, *fnr* and *resDE* mutations did not affect anaerobic YwfI formation in the presence of nitrate.

Upstream of *ywfI*, and transcribed in the opposite direction, the *pta* gene, encoding the fermentation enzyme acetyl-CoA:orthophosphate acetyltransferase (Pta), was localized. Pta catalyses phosphorylation of acetyl-CoA during fermentative acetate formation (Böck & Sawers, 1996). The 5′ region of *ywfI* shared with the fermentative gene *pta* could provide the basis for a coregulatory mechanism during the anaerobic adaptation process. The function and regulation of *ywfI* in combination with *pta* are subjects of ongoing research in our laboratory.

**Anaerobic induction of fructose-1,6-bisphosphate aldolase (FbaA) and dehydrolipoamide dehydrogenase (PhdD, Lpd)**

To investigate the consequences of changes in oxygen tension on the pattern of membrane proteins and membrane-associated proteins, blue native gel electrophoresis was combined with SDS gel electrophoresis in the second dimension (Schägger & von Jagow, 1991). We isolated and separated approximately 50 proteins which were visible after Coomassie blue staining. A reproducible oxygen-tension-dependent variation of the cellular concentration of three proteins was observed in five independent experiments (Fig. 2). One protein induced under all anaerobic conditions tested, and identified by N-terminal sequence determination, was fructose-1,6-bisphosphate aldolase (FbaA) (Table 1, Fig. 2). Anaerobic induction was found to be independent of regulation by *fnr* and *resDE* (Fig. 5b). Determination of the enzymic activity in the cytoplasmic and membrane fractions prepared from aerobically and anaerobically grown cells identified the majority of fructose-1,6-bisphosphate aldolase activity in the anaerobic membrane fraction (data not shown). Control of fructose-1,6-bisphosphate aldolase expression by oxygen tension could modulate the flux of the phosphorylated hexoses into glycolysis.

The second protein which was found to be anaerobically induced was identified as dehydrolipoamide dehydrogenase (PhdD, Lpd), the E3 subunit of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase. Similar to the situation with FbaA, a general anaerobic induction of PhdD formation independent of the presence of nitrate or nitrite was observed (Fig. 5c). Mutations of *fnr* and *resDE* did not change the observed induction (Fig. 5). In *E. coli*, *lpdA* was found to be subject to anaerobic derepression mediated by the two-component redox-regulatory system encoded by *arcA/B* (Quail et al., 1994; Cunningham et al., 1998). No obvious influence of *E. coli fnr* on *E. coli lpdA* expression was observed.

**Glycerol kinase (GlpK) and the potential NADH dehydrogenase (YjlD) are anaerobically repressed**

Two proteins showed a decrease in cellular concentration under all anaerobic conditions investigated. Due to the low resolution of the two-dimensional gels in the appropriate pH region presented in Fig. 1, the repression of GlpK is not visible there. On two-dimensional gels with a higher resolution in the area around pH 6 clear anaerobic GlpK repression was detectable (data not shown). The same degree of anaerobic GlpK repression is visible in Fig. 3 under conditions representing a combination of anaerobic adaptation and anaerobic stress response (see below). In agreement with these findings, glycerol kinase activity was found to be significantly decreased under anaerobic conditions (data not shown). The *glpK* gene forms an operon with the glycerol transporter gene *glpF* (Holmberg et al., 1990; Holmberg & Rutberg, 1989). The physiological reason and the molecular basis for anaerobic *glpK* repression will be the subject of future studies.

The analysis of the membrane-associated fraction using blue native gel electrophoresis revealed the repression of YjlD under all anaerobic conditions analysed (Figs 2 and 5d, Table 1). YjlD shares significant amino acid sequence identity with an *E. coli* NADH dehydrogenase encoded by *ndh*. This membrane-associated enzyme transfers electrons from NADH to membrane-localized electron-transport chains without direct participation in proton gradient formation (Meng et al., 1997). Similar to our findings for the *B. subtilis* protein, *E. coli Ndh* formation is repressed under anaerobic conditions. Transcriptional repression of the *E. coli ndh* gene is mediated by the redox regulator Fnr (Meng et al., 1997). However, the observed regulation of *B. subtilis* YjlD (Ndh) formation was independent of *fnr* and *resDE* (Fig. 5d). In agreement with these findings, no obvious *B. subtilis* Fnr-binding site was detected in the 5′ region of the *yjlD* gene. The regulatory mechanisms involved in anaerobic gene repression in *B. subtilis* are completely unknown. The identification of target genes described here should provide the basis for a detailed analysis of the molecular basis of this adaptation process.

**Anaerobic stress leads to a catabolic-response-like reaction**

When *B. subtilis* was directly shifted from aerobic conditions to strictly anaerobic conditions (degassed nitrate-containing medium, nitrogen atmosphere) a
growth lag phase of approximately 20 h was observed, indicating anaerobic stress by the absence of the enzymic systems required for anaerobic survival (Hoffmann et al., 1995). The protein patterns of cytoplasmic extracts prepared from cultures restarting growth under strictly anaerobic conditions were compared with those of extracts prepared from aerobic cultures. Multiple new proteins induced by anaerobic stress, in addition to proteins already found to be induced by anaerobiosis, were identified (Fig. 3). Again, the anaerobic induction of Hmp and the repression of GlpK were observed (Fig. 3). Since cultures only started to regrow in the presence of nitrate, protein patterns from fermentative cultures were not analysed and, consequently, no LctE induction was observed. However, induced proteins in addition to Hmp were identified. Four proteins involved in the metabolism of inositol (IolA, IolG, IolH, IolI), one in the utilization of melibiose (MelA), one aldehyde dehydrogenase (DhaS), and an NADH-dependent 6-phospho-α-ketogluconate (GlvA) were identified by their N-terminal sequences (Table 1). The iol genes involved in inositol utilization in B. subtilis and their catabolite-dependent regulation were recently described (Yoshida et al., 1997). In E. coli the melA gene is also under catabolite regulation (Okada et al., 1981; Liljestrom & Liljestrom, 1987). B. subtilis DhaS has 50% homology to an NAD· dependent aldehyde dehydrogenase from Bacillus stearothermophilus (Robinson et al., 1994). Finally, glvA encodes an α-glucosidase catalysing the hydrolysis of 6-phospho-α-glucosides including maltose 6-phosphate and trehalose 6-phosphate (Thompson et al., 1998).

In order to exclude stationary-phase effects on the observed expression patterns, control gels with extracts prepared from B. subtilis grown aerobically into stationary phase were analysed in parallel. None of the anaerobically induced proteins was found to be affected in its synthesis by the growth phase (data not shown). However, most of these proteins have in common that the corresponding genes are subject to catabolite repression. Therefore, one possible explanation for the observed anaerobic stress response is the release of a signal leading to a catabolite regulation (Hueck & Hillen, 1995; Kruger et al., 1996; Martin-Verstraete et al., 1995). In the absence of the electron acceptor oxygen and without appropriate anaerobic catabolic enzymes, ATP synthesis and NAD· regeneration should decrease drastically. Under these conditions growth ceased (Hoffmann et al., 1995). As a consequence, various metabolites, including potential signal molecules for a catabolite response, could accumulate, leading to the observed changes in protein synthesis. The influence of cellular NADH concentration on the activity of the B. subtilis catabolite regulator CcpA was recently described (Kim et al., 1998).

The relevance of the observed stress response for the environmental survival of B. subtilis is rather questionable, since, under natural conditions, the bacterium should usually not encounter this artificial stress situation.

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