Microbiology (2000), **146**, 97–105 Printed in Great Britain

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 (YjlD, Ndh), and anaerobic induction of fructose-1,6-bisphosphate aldolase (FbaA) and dehydrolipoamide 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 *narGHJI* 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

This paper is dedicated to Professor Dr R. K. Thauer, Marburg, on the occasion of his 60th birthday.

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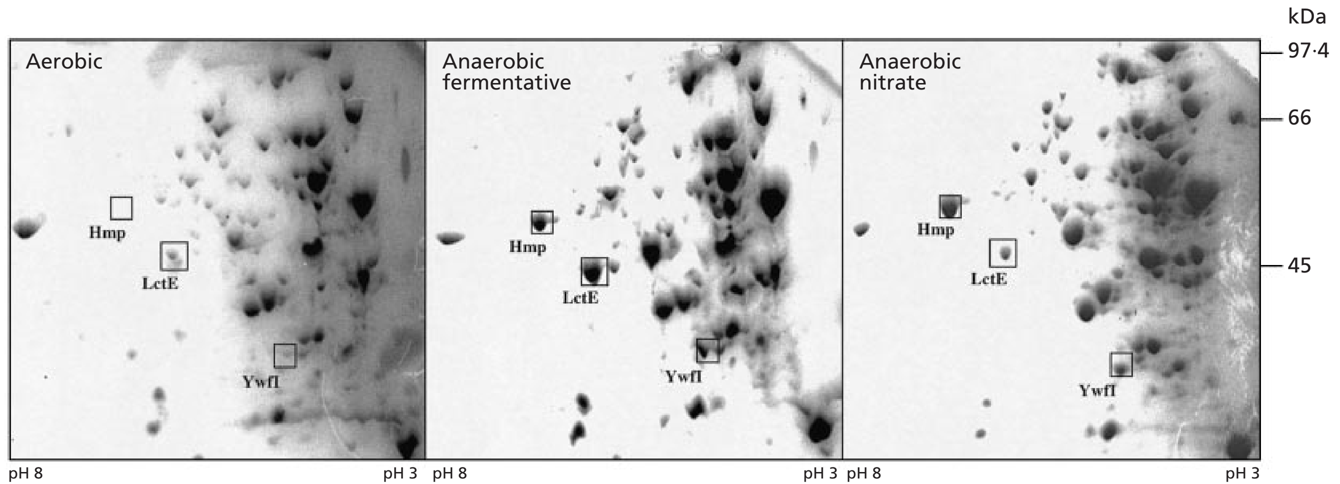

Fig. 1. Two-dimensional gel electrophoresis of cytoplasmic proteins extracted from *B. subtilis* grown under aerobic and anaerobic conditions in the presence and absence of the alternative electron acceptor nitrate (10 mM). N-terminal amino acid sequences of the indicated proteins were determined.

the same nitrite 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* P*spac-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 $NaNO_3$ as described before (Hoffmann *et al*., 1998). Cells were harvested, washed in 67 mM K_3PO_4 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 (BLOSUM 62 matrix) (Altschul *et al*., 1990; Moszer *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 1. Proteins responding to changes in environmental oxygen tension and to anaerobic stress

Protein	Function	N-terminal sequence	Accession no.
GlpK	Glycerol kinase (EC 2.7.1.30)	METTYLILSLDQGTTSDRAILFNKEG	P18157
Hmp	Flavohaemoglobin	MLDNKTIEIIKSTVPVLQQHGTIT	P49852
LctE	L-Lactate dehydrogenase (EC 1.1.1.27)	MNKHVNKKALIG	P13714
YwfI	Unknown	SEQQMTNEAAKT	P39645
YjlD (Ndh)	Unknown; similar to NADH dehydrogenase	SKHIVILGAG	–
FbaA	Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13)	PLVSMTEMLNTAK	P13243
PhdD (Lpd)	Dihydrolipoamide dehydrogenase, E3 subunit of pyruvate dehydrogenase/2-oxoglutarate dehydrogenase (EC 1.8.1.4)	VVGDFPIETDTL	P21880
MelA	α-D-Galactoside galactohydrolase (EC 3.2.1.22)	MKKITFIGAGSTIFAKNILGD	–
IolH	*myo*-Inositol catabolism	MKLALDPSMYRDDLTLEEMVYKTAE	P42418
IolG	*myo*-Inositol 2-dehydrogenase (EC 1.1.1.18)	MSLRIGVIGTGAIGKEHINRITNKL	P26935
DhaS	Aldehyde dehydrogenase	TSLTMQVTKYLETFLQGTKXLRI	–
IolI	*myo*-Inositol catabolism protein	MKLXFNEATTLANSNLKLDL	P42419
IolA	Methylmalonate-semialdehyde dehydrogenase (EC 1.2.1.27)	AEIRKLKNYINGE	P42412
GlvA	6-Phospho-α-glucosidase (EC 3.2.1.122)	MKKKSFLIVIAGXGXTF	P54716

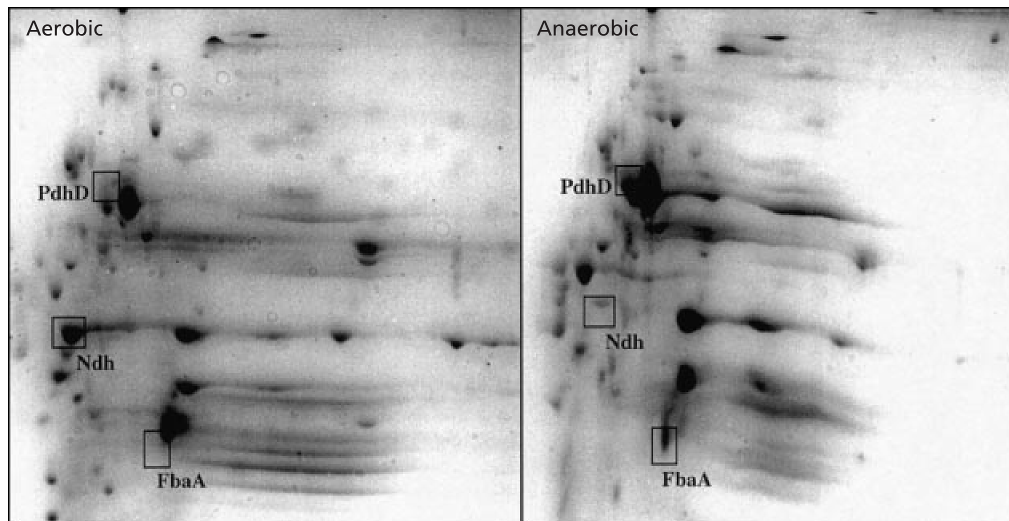

Fig. 2. Analysis of membrane proteins and membrane-associated proteins extracted from *B. subtilis* grown under aerobic and anaerobic conditions in the presence of 10 mM nitrate using blue native gel electrophoresis in the first dimension and SDS gel electrophoresis in the second dimension. N-terminal 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), dehydrolipoamide 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*.

Regulation of lactate dehydrogenase synthesis

Under anaerobic conditions in the absence of alternative electron acceptors the formation of lactate dehydrogenase (LctE) was found to be drastically increased (Figs 1 and 4a). The presence of nitrate greatly decreased the observed induction (Fig. 4a), whereas nitrite had no significant effect on anaerobic LctE production. To reconfirm these observations functionally, lactate dehydrogenase tests were performed. Low activity (25 units lactate dehydrogenase activity per g total cellular protein) was detected in extracts prepared from aerobically grown cells, whereas extracts from fermentatively grown cells yielded 17600 units. The presence of nitrate in the growth medium drastically reduced lactate dehydrogenase activity to 450 units, whereas the presence of nitrite in the growth media produced no comparable reduction (14900 units). The measured activities are in good agreement with the observed LctE protein amounts (Fig. 4a).

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

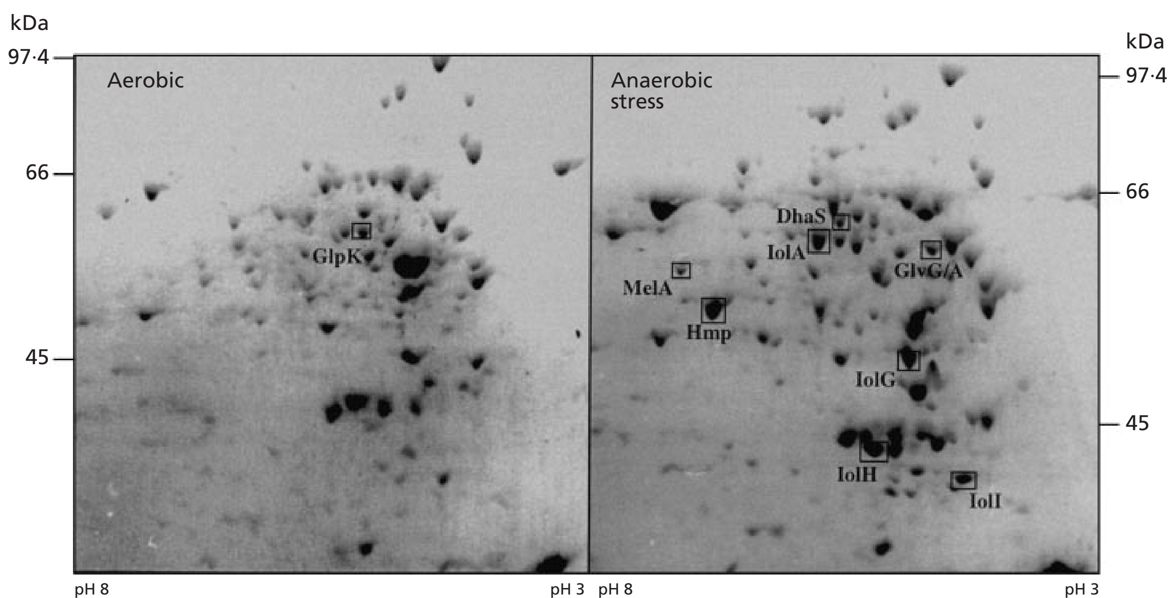

Fig. 3. Two-dimensional gel electrophoresis of cytoplasmic proteins extracted from *B. subtilis* grown under anaerobic stress. *B. subtilis* was grown under strictly aerobic conditions and shifted to strictly anaerobic conditions in the presence of 10 mM nitrate. N-terminal amino acid sequences of the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

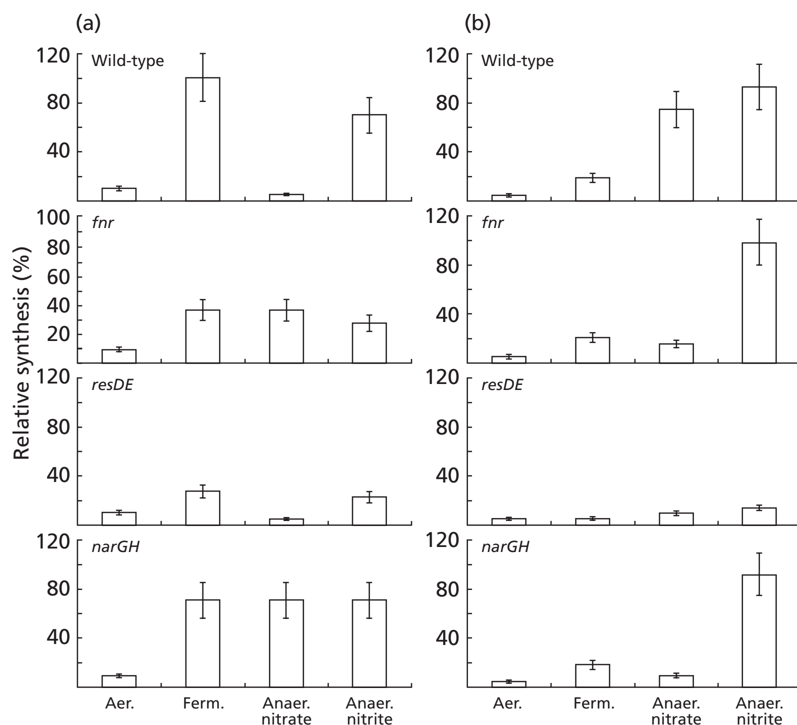

Fig. 4. Synthesis of (a) LctE and (b) Hmp in dependence on oxygen tension, the presence of alternative electron acceptors and mutations in regulatory genes. The cytoplasmic fraction of cell-free extracts prepared from *B. subtilis* JH642 (wild-type), THB2 (*fnr*), LAB2313 (*resDE*) and THB1 (*narGH*) 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.

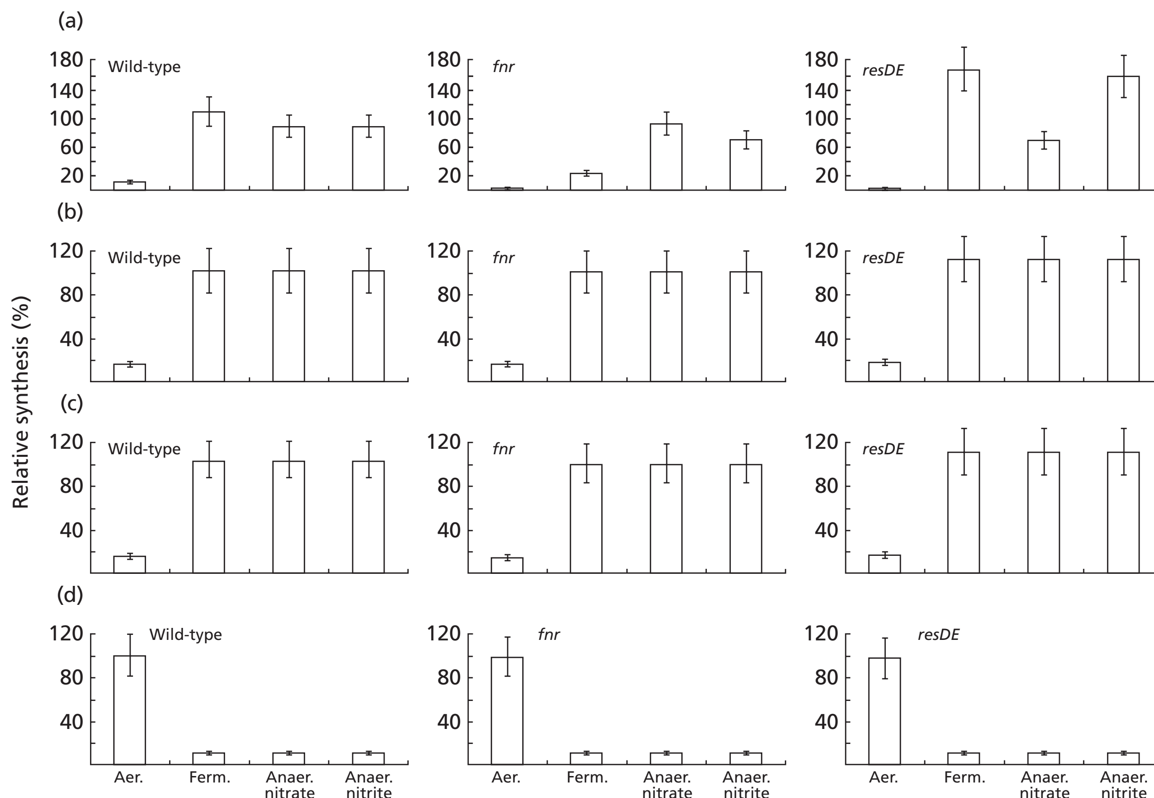

Fig. 5. Synthesis of (a) YwfI, (b) FbaA, (c) PhdD and (d) Ndh (YjlD) in dependence on oxygen tension, the presence of alternative electron acceptors and mutations in regulatory genes. The cytoplasmic (YwfI) and membrane-associated fractions (FbaA, PhdD, Ndh) of cell-free extracts prepared from *B. subtilis* JH642 (wild-type), THB2 (*fnr*), and LAB2313 (*resDE*) were 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 ($n = 3$).

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-glucosidase (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; Liljeström & Liljeström, 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; Krüger *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.

ACKNOWLEDGEMENTS

We thank Professor Dr R. K. Thauer (Max-Planck-Institut Marburg, Germany) for continuous support. We are indebted to Dr P. Graumann and Professor Dr M. Marahiel (Chemistry Department, University of Marburg, Germany) for many helpful discussions. We thank M. Kurz, Dr J. Rassow and Dr. M. Ryan (Biochemistry and Molecular Biology Department of the Medical School, University of Freiburg, Germany) for their introduction to blue native gel electrophoresis. This work was supported by grants of the Deutsche Forschungsgemeinschaft, the Max-Planck-Gesellschaft, the Institute of Organic Chemistry and Biochemistry of the University of Freiburg, and the Fonds der Chemischen Industrie.

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Received 27 August 1999; accepted 23 September 1999.