Genome-wide transcriptional profiling of the *Bacillus subtilis* cold-shock response

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The transcriptome of *Bacillus subtilis* was analysed at different time points (30, 60 and 90 min) after a temperature downshift from 37 to 18 °C using DNA macroarrays. This approach allowed the identification of around 50 genes exhibiting an increased mRNA level and around 50 genes exhibiting a decreased mRNA level under cold-shock conditions. Many of the repressed genes encode enzymes involved in the biosynthesis of amino acids, nucleotides and coenzymes, indicating metabolic adaptation of the cells to the decreased growth rate at the lower temperature. The strongest cold-inducible gene encodes fatty acid desaturase, which forms unsaturated fatty acids from saturated phospholipid precursors, thereby increasing membrane fluidity. The cold-shock-induced increase of mRNA levels of the classical cold-shock genes *cspB*, *cspC* and *cspD* could be verified. Furthermore, besides many genes encoding proteins of unknown function, some genes encoding ribosomal proteins were transcriptionally up-regulated, which points to an adaptive reprogramming of the ribosomes under cold-shock conditions. Interestingly, the amount of mRNA specified by the operon *ptb-bcd-buk-lpd-bkdA1-bkdA2-bkdB*, which encodes enzymes involved in degradation of branched-chain amino acids, also increases after a temperature downshift. As cells utilize the isoleucine and valine degradation intermediates α -methylbutyryl-CoA and isobutyryl-CoA for synthesis of branched-chain fatty acids, this finding reflects the adaptation of membrane lipid composition, ensuring the maintenance of appropriate membrane fluidity at low temperatures. The results of the DNA array analyses were verified for several selected genes by RNA slot-blot analysis and compared with two-dimensional PAGE analyses.

Keywords: temperature downshift, transcriptome, proteome, mRNA, DNA array

INTRODUCTION

In its natural environment, the surface layer of soil, the Gram-positive mesophilic bacterium *Bacillus subtilis* is exposed to drastic temperature changes. Recently, the following problems of mesophilic bacteria under cold-shock conditions have been addressed (Graumann & Marahiel, 1999): (a) low membrane fluidity, (b) unwinding of the DNA double helix due to increased superhelical density of the DNA, (c) reduced enzyme activities, (d) decreased initiation of translation due to stabilized secondary structures of the mRNAs, (e) adaptation of the ribosomes and (f) slower protein folding. Thus, a temperature downshift requires a dramatic adaptive cellular response which has to be

realized by global changes in the gene expression pattern.

Although synthesis of the majority of proteins decreases after a temperature downshift, the level of a specific group of proteins increases under these conditions in *B. subtilis* (Lottering & Streips, 1995; Graumann *et al.*, 1996). Using two-dimensional (2D) protein gel electrophoresis, it has been demonstrated that the synthesis of at least 37 proteins of *B. subtilis* increases after a temperature shift from 37 to 15 °C (Graumann *et al.*, 1996). It is supposed that these proteins play important roles in the adaptation of the cellular machinery to low-temperature conditions (Graumann & Marahiel, 1999). One of the highest accumulating protein groups of *B.*

subtilis after a temperature downshift is formed by CspB, CspC and CspD (Graumann *et al.*, 1996). It is assumed that these cold-shock proteins are able to bind mRNA and thus act as RNA chaperones, keeping critical mRNAs accessible for the ribosomes under low-temperature conditions (Graumann *et al.*, 1997; Schindler *et al.*, 1999). Furthermore, it has been suggested that cold-shock-induced proteins associated with translation, e.g. the ribosomal proteins S6 and L7/L12, are involved in the adaptation of ribosome function to low-temperature conditions (Graumann & Marahiel, 1999). There are also indications that prolyl isomerization represents a problem that cells have to cope with at low temperatures (Kandror & Goldberg, 1997; Graumann & Marahiel, 1999). Increasing levels of the peptidyl-prolyl *cis-trans* isomerases PpiB and Tig of *B. subtilis* in response to cold shock have been described by Graumann *et al.* (1996). Finally, it has been observed that the level of several enzymes required for general cellular metabolism, e.g. amino acid synthesis or glycolysis, increases after a temperature downshift (Graumann & Marahiel, 1999). It was suggested that the higher protein level compensates for the lower activity of these essential enzymes under low-temperature conditions.

It is supposed that the expression of most cold-shock-induced proteins is regulated mainly at the post-transcriptional and translational level. However, examples of regulation of cold-inducible genes at the transcriptional level have also been described. One example is the *des* gene of *B. subtilis* (Aguilar *et al.*, 1998). This gene encodes a $\Delta 5$ -lipid desaturase and exhibits one of the highest induction ratios during cold shock (Aguilar *et al.*, 1999). The *des* mRNA level strongly increases 30 min after a temperature downshift. Recently, it has been described that expression of *des* is regulated by a two-component signal transduction system composed of the sensor kinase DesK and the response regulator DesR (Aguilar *et al.*, 2001).

Besides direct transcriptional induction of cold-shock gene expression, mRNA stabilization after a temperature downshift has also been addressed as a mechanism for increased synthesis of cold-shock-induced proteins. It has been demonstrated that the overall mRNA half-life increases sixfold when *B. subtilis* cells are shifted from 37 to 20 °C (Aguilar *et al.*, 1999). For the genes of the major cold-shock proteins of *B. subtilis*, CspB and CspC, an approximately 40-fold increase in mRNA stability after a temperature downshift was shown (Kaan *et al.*, 1999), which is significantly greater than the increase in bulk mRNA stability. A similar dramatic stabilization has been described for the mRNA of the major cold-shock protein of *Escherichia coli*, CspA (Brandi *et al.*, 1996; Goldenberg *et al.*, 1996; Fang *et al.*, 1997). Thus, besides the translational regulation of the synthesis of cold-shock proteins, transcriptional and post-transcriptional events are of importance for the adaptational response of bacterial cells under cold-shock conditions.

The aim of this study was to obtain a more comprehensive view of the cold-shock response of *B. subtilis*.

2D protein gel electrophoresis allows analysis of distinct groups of proteins from various cellular compartments with some limitations. However, global gene expression profiling by means of DNA arrays allows the transcriptional analysis of all genes of a specific genome at one defined time point. We analysed the transcriptome of *B. subtilis* after a temperature downshift from 37 to 18 °C. This approach allowed the identification of 46 genes exhibiting increased mRNA levels and 52 genes exhibiting decreased mRNA levels under cold-shock conditions. The contribution of altered mRNA levels to the varying amounts of proteins after cold shock was analysed by comparing transcriptome and proteome data.

METHODS

Bacterial strains and growth conditions. *B. subtilis* 168 (*trpC2*) was used in this study (Anagnostopoulos & Spizizen, 1961). This strain was cultivated under vigorous agitation at 37 and 18 °C in a synthetic minimal medium supplemented with glucose (0.2%) and tryptophan (50 µg ml⁻¹) (Stülke *et al.*, 1993). The cold shock was carried out as a temperature downshift from 37 to 18 °C at an OD₅₀₀ of about 0.4.

Growth experiments were performed with *B. subtilis* strains BFS4469, BFS4564, YHEAD, BFS4794 and BFS4226 carrying pMUTIN integrations (Vagner *et al.*, 1998) in *ydeB*, *ydjO*, *yheA*, *yqgA* and *yacK*, respectively. Strains were cultivated in the minimal medium described above as well as in Spizizen's minimal medium (Spizizen, 1958) supplemented with 0.2% glucose and 0.08 mM tryptophan. Erythromycin and lincomycin were added to final concentrations of 1 and 10 µg ml⁻¹, respectively.

RNA extraction. For RNA extraction, *B. subtilis* cells were disrupted in liquid-nitrogen-chilled vessels using a Micro Dismembrator S (Braun Biotech International) for 2 min at 2600 r.p.m. Total RNA was isolated by using the single step method as described by Chomczynski & Sacchi (1987). The RNA pellet was resuspended in sterile water and the RNA concentration was determined spectrophotometrically.

Preparation of labelled cDNA and hybridization to macroarrays. Reverse transcription and hybridization of the purified labelled target cDNA to *B. subtilis* nylon arrays were performed as described by Wiegert *et al.* (2001) with the following modifications: 10 µg total RNA was used, and macroarrays and specific cDNA primers were purchased from Eurogentec. Hybridization signals were detected by exposure of the arrays to a storage phosphor screen (Molecular Dynamics). Each analysis was carried out twice using two independently isolated RNA preparations and two different array batches.

Data analysis. After scanning of the phosphor screens using a Storm 840 Phosphor Imager (Molecular Dynamics), the resulting images were analysed using ArrayVision 5.1 software (Imaging Research). Calculation of normalized intensity values of the individual spots was performed using the overall-spot-normalization function of ArrayVision. To avoid extreme intensity ratios for genes close to or below the detection limit, signal intensity values corresponding to a signal to noise (*S/N*) ratio < 1.0 were scaled up to a value corresponding to an *S/N* ratio = 1.0. Further analyses were carried out using GeneSpring 3.2.12 software (Silicon Genetics). Genes exhibiting *S/N* ratios ≥ 3 under at least one growth condition

Table 1. List of primers used for the generation of PCR products

Gene	Primer	Sequence 5'→3'
<i>des</i>	T7DESFOR	CGTATCTTCAATGGCAGCAC
	T7DESREV	<u>CTAATACGACTCACTATAGGGAGACAAAGATCAGACAG</u>
<i>cspB</i>	T7CSPBFOR	CGGATTCATCGAAGTAGAAGG
	T7CSPBREV	<u>CTAATACGACTCACTATAGGGAGAACGTTAGCAGCTTGTGGTCCG</u>
<i>cspC</i>	T7CSPCFOR	CGAACGCGAAAAATGGAGACG
	T7CSPCREV	<u>CTAATACGACTCACTATAGGGAGAACGTTAGCAGCTTGAG</u>
<i>purA</i>	T7PURAFOR	ATGTCTTCAGTAGTTGTAGTA
	T7PURAREV	<u>CTAATACGACTCACTATAGGGAGATTAGTTCGCACGGTACACACTG</u>
<i>yydG</i>	T7YYDGFOR	GAGAGTTAGTAACTG
	T7YYDGREV	<u>CTAATACGACTCACTATAGGGAGACCCAATTCCTCTAAA</u>
<i>yvcE</i>	T7YVCEFOR	GACATTAACGATAAG
	T7YVCEREV	<u>CTAATACGACTCACTATAGGGAGAAAGTTGTGCGCCGTTTG</u>
<i>apt</i>	T7APTFOR	GGATTACCCGAAAGA
	T7APTREV	<u>CTAATACGACTCACTATAGGGAGACTGCCGTCAAGGTAA</u>
<i>yvfG</i>	T7YVGFOR	CCGTCCCTTATTTTA
	T7YVFGREV	<u>CTAATACGACTCACTATAGGGAGACTCCACGTTTGACTT</u>
<i>mntA</i>	T7MNTAFOR	TTGCGACCTTTGCTTTAACG
	T7MNTAREV	<u>CTAATACGACTCACTATAGGGAGATCTTTTGTGCCCTTTTCACC</u>

* The T7 RNA polymerase recognition sequence of the reverse primers is underlined.

were considered to be significantly expressed. All genes specifying signals below this significance threshold were excluded from further data analysis. Then, the mean of the normalized intensity values of the duplicate spots of each gene was used to calculate the expression level ratios. Induction or repression ratios ≥ 2 in both experiments were considered as significant.

Final evaluation of the macroarray data included the consideration of putative operons derived from the genome sequence using the SubtiList database (<http://genolist.pasteur.fr/SubtiList/>) as well as previously known transcriptional units. Genes exhibiting significant expression ratios were checked for their transcriptional organization. If these genes were members of polycistronic transcriptional units, further members of these operons were also included in the resulting tables even if their expression parameters did not fulfil the significance criteria.

RNA slot-blot analysis. Digoxigenin-labelled probes were obtained by *in vitro* transcription with T7 RNA polymerase using PCR-generated DNA fragments as templates. Primers used for PCR are listed in Table 1. Reverse primers contained the T7 RNA polymerase recognition sequence. The probes were generated with the Dig Chem Link Labelling and Detection Set (Roche Diagnostics).

Slot-blot analysis was performed with 0.5 or 10 µg total RNA by using standard methods (Wetzstein *et al.*, 1992). For quantification of specific mRNAs, serial dilutions of total RNA in 10 × SSC were transferred to a positively charged nylon membrane and hybridized with the appropriate gene-specific probe.

Hybridization signals were detected according to the manufacturer's instructions (Roche Diagnostics). Chemilumino-graphs were analysed with a Lumi-Imager and the Lumi-Analyse-software package (Boehringer Mannheim). Signals

corresponding to mRNA samples taken from *B. subtilis* cells at 37 °C served as standards and were set to 1.

2D PAGE analyses. Pulse labelling of the bacterial culture for 5 min with 10 µCi L-[³⁵S]methionine ml⁻¹ during exponential growth was carried out as described by Schmid *et al.* (1997). After sonication of the harvested cells, the amount of protein was determined by using Roti-Nanoquant (Roth). Crude protein extracts (50 µg protein) were loaded onto IPG strips for the first dimension of 2D gel electrophoresis (Völker *et al.*, 1994). The second dimension was carried out as described by Bernhardt *et al.* (1997). After fixation and silver staining, the wet gels were scanned with a Hewlett Packard ScanJet 6000 in transmission mode at a resolution of 300 d.p.i. and an 8 bit/256 greys colour depth. For autoradiography of the radiolabelled protein pattern (after separation of 2 million c.p.m.), the dried gels were exposed to storage phosphor screens (Molecular Dynamics Storage Phosphor Screen; 20 × 25 cm) for 1–7 h and scanned with a STORM 840 Phosphor Imager (Molecular Dynamics) at a resolution of 200 µm and a colour depth of 16 bit/65 536 greys.

For computer-aided analysis of the gels, Delta2D 1.1 software (Decodon) was used as described by Bernhardt *et al.* (1999). The protein spots were relocated from the Sub2D database (Bernhardt & Werner, 1999).

RESULTS AND DISCUSSION

Transcriptome analysis: genes exhibiting significantly decreased mRNA levels

For the transcriptome analysis of cold-shocked *B. subtilis* cells, samples were taken at 30, 60 and 90 min after a temperature downshift from 37 to 18 °C. The growth rate of the cells decreased after the cold shock from 0.8 h⁻¹ at 37 °C to 0.1 h⁻¹ at 18 °C. The DNA array approach revealed 52 genes organized in

Table 2. List of *B. subtilis* genes exhibiting significantly decreased mRNA levels under cold-shock conditions determined by DNA macroarray analysis

The table shows the calculated mRNA ratios for the individual genes averaged from two parallel experiments, the partially putative transcriptional organization and the functions of the encoded gene products, also partially putative. If the calculated ratios at one given time point fulfil the strongest significance criteria (signal values threefold above the cut-off value and ratios differing at least twofold from the wild-type), the ratios are given in bold type. The sd of the mRNA ratios is presented in parentheses. Based on the proteome analyses, a decrease in the level of the corresponding protein is indicated by (–) and an increase is indicated by (+).

Gene	30 min cold shock	60 min cold shock	90 min cold shock	Transcriptional organization	Gene product function (partially putative)
Cell-wall-associated functions					
<i>wapA</i>	2·8 (0·4)	5·9 (0·9)	3·2 (0·7)	<i>wapA-yxxG</i>	Cell-wall-associated protein precursor
<i>yxxG</i>	4·8 (0·7)	5·3 (1·2)	8·3 (1·9)	<i>wapA-yxxG</i>	Unknown function
<i>ywtD</i>	2·1 (0·5)	5·6 (0·9)	3·5 (0·4)	<i>ywtD</i>	Similar to murein hydrolase
Transport/binding proteins and lipoproteins					
<i>opuCA</i>	1·4 (0·4)	4·4 (0·9)	4·8 (0·8)	<i>opuCC-opuCB-opuCA-opuCD</i>	Glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)
<i>opuCB</i>	1·2 (0·4)	4·2 (0·8)	4·6 (0·9)	<i>opuCC-opuCB-opuCA-opuCD</i>	Glycine betaine/carnitine/choline ABC transporter (membrane protein)
<i>opuCC</i>	1·5 (0·3)	3·7 (0·9)	4·6 (1·2)	<i>opuCC-opuCB-opuCA-opuCD</i>	Glycine betaine/carnitine/choline ABC transporter (osmoprotectant-binding protein)
<i>opuCD</i>	–	–	–	<i>opuCC-opuCB-opuCA-opuCD</i>	Glycine betaine/carnitine/choline ABC transporter (osmoprotectant-binding protein)
<i>yocS</i>	1·8 (0·4)	5·3 (1·2)	4·0 (1·3)	<i>yocS</i>	Similar to sodium-dependent transporter
<i>yodF</i>	2·0 (0·6)	3·5 (0·7)	2·7 (0·9)	<i>yodF</i>	Similar to proline permease
Amino acid biosynthesis					
<i>ilvB</i>	1·4 (–) (0·4)	3·9 (–) (1·4)	0·7 (–) (0·3)	<i>ilvB-ilvN-ilvC-leuA-leuB-leuC-leuD</i>	Acetolactate synthase (large subunit)
<i>ilvN</i>	2·0 (0·7)	5·6 (1·9)	4·2 (1·7)	<i>ilvB-ilvN-ilvC-leuA-leuB-leuC-leuD</i>	Acetolactate synthase (small subunit)
<i>ilvC</i>	1·3 (+) (0·2)	4·4 (+) (1·2)	2·6 (+) (1·5)	<i>ilvB-ilvN-ilvC-leuA-leuB-leuC-leuD</i>	Ketol-acid reductoisomerase
<i>leuA</i>	2·5 (–) (0·6)	8·3 (–) (3·1)	4·4 (–) (2·3)	<i>ilvB-ilvN-ilvC-leuA-leuB-leuC-leuD</i>	2-Isopropylmalate synthase
<i>leuB</i>	1·9 (–) (0·3)	7·7 (–) (4·1)	4·6 (–) (1·4)	<i>ilvB-ilvN-ilvC-leuA-leuB-leuC-leuD</i>	3-Isopropylmalate dehydrogenase
<i>leuC</i>	1·7 (–) (0·5)	5·0 (–) (2·1)	2·2 (–) (0·9)	<i>ilvB-ilvN-ilvC-leuA-leuB-leuC-leuD</i>	3-Isopropylmalate dehydratase (large subunit)
<i>leuD</i>	1·4 (0·2)	4·3 (1·4)	2·6 (0·8)	<i>ilvB-ilvN-ilvC-leuA-leuB-leuC-leuD</i>	3-Isopropylmalate dehydratase (small subunit)
<i>ilvD</i>	2·2 (–) (0·7)	6·7 (–) (1·5)	2·1 (–) (0·6)	<i>ilvD</i>	Dihydroxyacid dehydratase
<i>metC</i>	2·9 (1·3)	3·9 (0·7)	4·0 (0·8)	<i>metC</i>	Cobalamin-independent methionine synthase
Metabolism of nucleotides					
<i>guaB</i>	2·1 (–) (0·3)	4·2 (–) (0·7)	2·6 (–) (0·3)	<i>guaB</i>	Inosine monophosphate dehydrogenase
<i>purE</i>	2·4 (0·4)	1·0 (0·2)	2·6 (0·7)	<i>purE-purK-purB-purC-yexA-purL-purQ-purF-purM-purN-purH-purD</i>	Purine biosynthesis: phosphoribosylaminoimidazole carboxylase I
<i>purK</i>	0·9 (0·0)	1·8 (0·3)	2·1 (0·2)	<i>purE-purK-purB-purC-yexA-purL-purQ-purF-purM-purN-purH-purD</i>	Purine biosynthesis: phosphoribosylaminoimidazole carboxylase II

Table 2. (cont.)

Gene	30 min cold shock	60 min cold shock	90 min cold shock	Transcriptional organization	Gene product function (partially putative)
<i>purB</i>	1·6 (0·1)	2·6 (0·1)	2·6 (0·4)	<i>purE-purK-purB-purC-yexA-purL-purQ-purF-purM-purN-purH-purD</i>	Purine biosynthesis: adenylosuccinate lyase
<i>purC</i>	1·3 (0·3)	4·2 (0·6)	4·8 (0·9)	<i>purE-purK-purB-purC-yexA-purL-purQ-purF-purM-purN-purH-purD</i>	Purine biosynthesis: phosphoribosylaminoimidazole succinocarboxamide synthetase
<i>yexA</i>	5·9 (2·7)	2·5 (0·6)	4·8 (1·4)	<i>purE-purK-purB-purC-yexA-purL-purQ-purF-purM-purN-purH-purD</i>	Unknown function
<i>purL</i>	1·8 (0·4)	5·0 (1·4)	2·6 (0·5)	<i>purE-purK-purB-purC-yexA-purL-purQ-purF-purM-purN-purH-purD</i>	Purine biosynthesis: phosphoribosylformylglycinamide synthetase II
<i>purQ</i>	1·3 (0·2)	5·6 (1·2)	0·6 (0·4)	<i>purE-purK-purB-purC-yexA-purL-purQ-purF-purM-purN-purH-purD</i>	Purine biosynthesis: phosphoribosylformylglycinamide synthetase I
<i>purF</i>	2·0 (—) (0·2)	3·5 (—) (0·4)	4·6 (—) (0·5)	<i>purE-purK-purB-purC-yexA-purL-purQ-purF-purM-purN-purH-purD</i>	Purine biosynthesis: glutamine phosphoribosylpyrophosphate amidotransferase
<i>purM</i>	1·6 (0·1)	1·6 (0·3)	1·8 (0·2)	<i>purE-purK-purB-purC-yexA-purL-purQ-purF-purM-purN-purH-purD</i>	Purine biosynthesis: phosphoribosylaminoimidazole synthetase
<i>purN</i>	1·7 (0·2)	8·3 (4·1)	5·3 (2·7)	<i>purE-purK-purB-purC-yexA-purL-purQ-purF-purM-purN-purH-purD</i>	Purine biosynthesis: phosphoribosylglycinamide formyltransferase
<i>purH</i>	1·4 (0·1)	5·9 (2·2)	5·0 (1·6)	<i>purE-purK-purB-purC-yexA-purL-purQ-purF-purM-purN-purH-purD</i>	Purine biosynthesis: inosine monophosphate cyclohydrolase
<i>purD</i>	1·0 (0·1)	1·8 (0·3)	3·2 (1·3)	<i>purE-purK-purB-purC-yexA-purL-purQ-purF-purM-purN-purH-purD</i>	Purine biosynthesis: phosphoribosylglycinamide synthetase
<i>ymaA</i>	1·5 (0·1)	2·7 (0·3)	2·9 (0·5)	<i>ymaA-nrdE-nrdF-ymaB</i>	Unknown function
<i>nrdE</i>	2·2 (0·2)	4·4 (0·3)	4·6 (0·9)	<i>ymaA-nrdE-nrdF-ymaB</i>	Ribonucleoside diphosphate reductase (major subunit)
<i>nrdF</i>	—	—	—	<i>ymaA-nrdE-nrdF-ymaB</i>	Ribonucleoside diphosphate reductase (minor subunit)
<i>ymaB</i>	—	—	—	<i>ymaA-nrdE-nrdF-ymaB</i>	Unknown function
Metabolism of coenzymes					
<i>thiA</i>	1·4 (—) (0·1)	7·7 (—) (4·2)	5·3 (—) (2·6)	<i>thiA</i>	Biosynthesis of the pyrimidine moiety of thiamine (thiamine biosynthesis)
<i>bioW</i>	1·3 (0·1)	4·4 (1·4)	2·5 (0·5)	<i>bioW-bioA-bioF-bioD-bioB-bioI</i>	Biotin biosynthesis: 6-carboxyhexanoate-CoA ligase
<i>bioA</i>	1·3 (0·2)	4·5 (1·5)	2·6 (0·4)	<i>bioW-bioA-bioF-bioD-bioB-bioI</i>	Biotin biosynthesis: adenosylmethionine-8-amino- 7-oxononanoate aminotransferase
<i>bioF</i>	1·2 (0·1)	4·4 (1·4)	2·8 (0·5)	<i>bioW-bioA-bioF-bioD-bioB-bioI</i>	Biotin biosynthesis: 8-amino-7-oxononanoate synthase
<i>bioD</i>	1·2 (0·1)	4·6 (1·5)	3·1 (0·6)	<i>bioW-bioA-bioF-bioD-bioB-bioI</i>	Biotin biosynthesis: dethiobiotin synthetase
<i>bioB</i>	1·2 (0·1)	4·1 (1·3)	2·9 (0·6)	<i>bioW-bioA-bioF-bioD-bioB-bioI</i>	Biotin biosynthesis: biotin synthetase
<i>bioI</i>	1·2 (0·1)	4·0 (1·2)	2·9 (0·7)	<i>bioW-bioA-bioF-bioD-bioB-bioI</i>	Biotin biosynthesis: cytochrome P450-like enzyme

Table 2. (cont.)

Gene	30 min cold shock	60 min cold shock	90 min cold shock	Transcriptional organization	Gene product function (partially putative)
Protein synthesis					
<i>ykrT</i>	1·4 (—) (0·1)	4·0 (—) (1·4)	2·7 (—) (0·6)	<i>ykrT-ykrS</i>	Unknown function
<i>ykrS</i>	1·5 (—) (0·2)	4·2 (—) (1·5)	3·3 (—) (0·9)	<i>ykrT-ykrS</i>	Similar to initiation factor eIF-2B (α subunit)
Detoxification					
<i>ahpC</i>	1·6 (0·1)	4·4 (1·6)	4·0 (1·4)	<i>ahpC-ahpF</i>	Alkyl hydroperoxide reductase (small subunit)
<i>ahpF</i>	1·7 (0·2)	4·6 (1·7)	3·9 (1·2)	<i>ahpC-ahpF</i>	Alkyl hydroperoxide reductase (large subunit) and NADH dehydrogenase
Unknown function					
<i>yxbB</i>	1·8 (0·2)	11·1 (5·3)	12·5 (0·9)	<i>yxbB-yxbA-yxnB-asnH-yxaM</i>	Similar to proteins of unknown function
<i>yxbA</i>	—	—	—	<i>yxbB-yxbA-yxnB-asnH-yxaM</i>	Unknown function
<i>yxnB</i>	1·8 (0·2)	6·7 (2·7)	9·1 (5·4)	<i>yxbB-yxbA-yxnB-asnH-yxaM</i>	Unknown function
<i>asnH</i>	1·6 (0·2)	5·9 (2·1)	5·9 (2·3)	<i>yxbB-yxbA-yxnB-asnH-yxaM</i>	Asparagine synthetase
<i>yxaM</i>	1·9 (0·3)	7·7 (3·9)	6·3 (1·6)	<i>yxbB-yxbA-yxnB-asnH-yxaM</i>	Similar to antibiotic resistance protein
<i>yjbC</i> (—)	5·6 (—) (0·4)	2·4 (—) (0·4)	1·9 (—) (0·4)	<i>yjbC</i>	Unknown function

17 transcriptional units which exhibited significantly decreased mRNA levels (Table 2).

Most of the proteins encoded by the 52 down-regulated genes fulfil metabolic functions. The *ilvBHCleuABCD* operon (Grandoni *et al.*, 1992) as well as the monocistronic *ilvD* gene encode enzymes involved in the biosynthesis of branched-chain amino acids. The *metC* gene, encoding cobalamin-independent methionine synthase, as well as the bicistronic *ykrTS* operon, belong to the S-box regulon encompassing genes that are mainly involved in sulfate assimilation and methionine biosynthesis (Grundy & Henkin, 1998). Whereas the *ykrT* gene product most probably plays a role in the pathway that generates methionine from 5'-methylthioadenosine, a by-product of polyamine biosynthesis, the *ykrS* gene product shows similarity to the eukaryotic translation initiation factor eIF-2B (Murphy *et al.*, 2002). In all these cases, the maximal decrease in the mRNA levels was observed 60 min after the cold shock. The *asnH* gene encoding asparagine synthetase lies within the pentacistronic *yxbB-yxbA-yxnB-asnH-yxaM* operon which exhibited the strongest decreased expression obtained in this study. Expression of the *guaB* gene encoding the inosine-monophosphate dehydrogenase involved in biosynthesis of GMP (Saxild & Nygaard, 1988) was maximally decreased 60 min after the cold shock. On average, the large *purEKBC-yexA-purLQFMNHD* operon encoding purine biosynthesis enzymes (Ebböle & Zalkin, 1987) also showed a reduced transcript level under cold-shock conditions, although the mRNA level ratios of the single genes were more heterogeneous compared to the genes mentioned above. In the case of the tetracistronic *ymaA-nrdE-nrdF-ymaB* operon, which encodes the major and the minor subunit of ribonucleoside-diphosphate reductase (Scotti *et al.*, 1996) and exhibited a continuous decreasing mRNA level after cold shock, the signal values obtained for the two distal genes of the operon were below the significance threshold, therefore allowing no reliable quantification. Finally, the *bioWAFDBI* operon, encoding the biotin biosynthetic enzymes (Bower *et al.*, 1996), and the monocistronic *thiC* gene, whose product is involved in the biosynthesis of the pyrimidine moiety of thiamine (Begley *et al.*, 1999), showed the strongest decrease in their mRNA levels 60 min after cold shock.

Besides genes encoding proteins involved in amino acid biosynthesis, nucleotide and coenzyme metabolism, other down-regulated genes can be functionally classified in different groups. The mRNA levels of the *opuCACBCCCD* operon strongly decreased 60 min after the temperature downshift. This operon encodes a glycine betaine/carnitine/choline ABC transporter involved in osmoprotection (Kappes *et al.*, 1999). The monocistronic *yocS* and *yodF* genes most probably also encode transport/binding proteins, in particular a sodium-dependent transporter and a proline permease, respectively. Both genes revealed maximally decreased mRNA levels 60 min after the temperature downshift.

Two down-regulated transcriptional units specify

proteins fulfilling cell-wall-associated functions: the bicistronic *wapA-yxxG* operon and the monocistronic *ywtD* gene. Whereas *wapA* encodes a cell-wall-associated protein precursor (Foster, 1993), the function of the co-transcribed *yxxG* gene is unknown. Due to its sequence similarity, the gene product of *ywtD* most probably represents a murein hydrolase.

The bicistronic *ahpC-ahpF* operon, encoding the small and large subunits of the alkyl hydroperoxide reductase, was also strongly down-regulated by 60 min after cold shock. This enzyme fulfils an essential function in detoxification under conditions of oxidative stress (Antelmann *et al.*, 1996). The transcript level of the *yjbC* gene encoding a general stress protein of unknown function (Petersohn *et al.*, 2001) exhibited the maximal decrease by 30 min after cold shock.

The physiological function of the down-regulated genes is partially evident. In the case of *ywtD*, which most probably encodes a murein hydrolase, the reduction of the mRNA level could ensure the adjustment of the enzyme amount to the reduced growth rate under cold-shock conditions. Murein hydrolases are essentially involved in cell growth and cell division. Similarly, reduced expression of *wapA*, encoding a cell-wall-associated protein precursor (Foster, 1993), could function to adapt the amount of this large membrane protein to the general decline in membrane- and cell-wall-located functions during growth in the cold. The down-regulation of the *ahpC-ahpF* operon could reflect the lower activity of the respiratory chain enzymic components at low temperatures, resulting in a reduced production of potentially toxic, highly reactive side products of respiratory chain reactions.

The reduced expression of the following transcriptional units specifying biosynthetic enzymes can also be interpreted as consequence of the lower growth rate after cold shock: *bioWAFDBI*, *thiA*, *ymaA-nrdEF-ymaB*, *purEKBC-yexA-purLQFMNHD*, *guaB*, *metC*, *ilvD* and *ilvBHCleuABCD*. All encoded enzymes from these genes are involved in major biosynthetic processes, such as the biosynthesis of amino acids, nucleotides and coenzymes. Therefore, it is obvious to speculate that the observed decreased mRNA levels reflect the cellular reaction to the reduced growth rate at lower temperature.

The physiological relevance of the down-regulation of the glycine betaine ABC transporter *opuCACBCCCD* as well as of *yocS* and *yodF*, encoding transporter/permease proteins, remains unclear. The *opuC* operon functions in coping with osmotic stress (Kappes *et al.*, 1999). The operon encodes an ABC transporter responsible for the uptake of the compatible solutes glycine betaine, carnitine and choline. It is transcriptionally induced under conditions of increased osmolarity, but also expressed at a low level under conditions of moderate osmolarity. The adaptive effect of a lowering of the basal expression of the *opuC* operon under cold-shock conditions is not obvious.

Table 3. List of *B. subtilis* genes exhibiting significantly increased mRNA levels under cold-shock conditions determined by DNA macroarray analysis

The table shows the calculated mRNA ratios for the individual genes averaged from two parallel experiments, the partially putative transcriptional organization and the functions of the encoded gene products, also partially putative. If the calculated ratios at one given time point fulfil the strongest significance criteria (signal values threefold above the cut-off value and ratios differing at least twofold from the wild-type), the ratios are given in bold type. The SD of the mRNA ratios is presented in parentheses. Based on the proteome analyses, a decrease of the level of the corresponding protein is indicated by (–) and an increase is indicated by (+).

Gene	30 min cold shock	60 min cold shock	90 min cold shock	Transcriptional organization	Gene product function (partially putative)
Cell-wall-associated functions					
<i>yvcE</i>	2.7 (0.5)	4.9 (0.9)	4.6 (0.5)	<i>yvcE</i>	Similar to cell-wall-binding protein
Transport/binding proteins and lipoproteins					
<i>mntA</i>	1.7 (0.1)	2.5 (0.1)	2.2 (0.2)	<i>mntA-mntB-mntC</i>	Manganese ABC transporter (membrane protein)
<i>mntB</i>	2.6 (0.2)	4.4 (0.9)	4.0 (0.6)	<i>mntA-mntB-mntC</i>	Manganese ABC transporter (ATP-binding protein)
<i>mntC</i>	2.1 (0.1)	3.5 (0.7)	2.9 (0.5)	<i>mntA-mntB-mntC</i>	Manganese ABC transporter (membrane protein)
<i>yydF</i>	1.6 (0.3)	2.3 (0.6)	2.9 (0.4)	<i>yydF-yydG-yydH-yddI-yddJ</i>	Unknown function
<i>yydG</i>	1.3 (0.1)	1.7 (0.3)	2.0 (0.6)	<i>yydF-yydG-yydH-yddI-yddJ</i>	Unknown function
<i>yydH</i>	1.5 (0.1)	1.9 (0.2)	2.3 (0.4)	<i>yydF-yydG-yydH-yddI-yddJ</i>	Unknown function
<i>yddI</i>	1.4 (0.1)	1.7 (0.4)	2.1 (0.3)	<i>yydF-yydG-yydH-yddI-yddJ</i>	Similar to ABC transporter (ATP-binding protein)
<i>yydJ</i>	1.0 (0.1)	1.5 (0.2)	2.2 (0.4)	<i>yydF-yydG-yydH-yddI-yddJ</i>	Unknown function
Metabolism of carbohydrates					
<i>fbaA</i>	1.2 (–) (0.1)	1.2 (–) (0.1)	5.3 (–) (2.9)	<i>fbaA</i>	Fructose-1,6-bisphosphate aldolase
<i>yrpG</i>	1.6 (0.2)	2.9 (0.3)	1.2 (0.1)	<i>yrpG</i>	Similar to sugar-phosphate dehydrogenase
Metabolism of nucleotides					
<i>purA</i>	2.6 (–) (0.3)	5.0 (–) (1.9)	4.0 (–) (1.2)	<i>purA</i>	Adenylosuccinate synthetase
Metabolism of lipids					
<i>des</i>	2.5 (0.2)	10.3 (1.6)	3.3 (0.4)	<i>des</i>	Membrane phospholipid desaturase
Metabolism of amino acids					
<i>bkdR</i>	–	–	–	<i>bkdR-ptb-bcd-buk-lpd-bkdA1-bkdA2-bkdB</i>	Positive transcriptional activator of the isoleucine and valine degradation pathway
<i>ptb</i>	2.1 (0.2)	3.1 (0.4)	3.9 (0.8)	<i>bkdR-ptb-bcd-buk-lpd-bkdA1-bkdA2-bkdB</i>	Phosphate butyryl coenzyme A transferase
<i>bcd</i>	2.3 (0.4)	3.6 (1.6)	4.0 (1.5)	<i>bkdR-ptb-bcd-buk-lpd-bkdA1-bkdA2-bkdB</i>	Leucine dehydrogenase
<i>buk</i>	1.3 (0.1)	1.6 (0.4)	2.0 (0.4)	<i>bkdR-ptb-bcd-buk-lpd-bkdA1-bkdA2-bkdB</i>	Butyrate kinase
<i>lpd</i>	1.3 (0.1)	2.0 (0.2)	2.4 (0.2)	<i>bkdR-ptb-bcd-buk-lpd-bkdA1-bkdA2-bkdB</i>	Butyrate dehydrogenase
<i>bkdA</i>	2.5 (0.3)	4.0 (0.7)	4.9 (1.6)	<i>bkdR-ptb-bcd-buk-lpd-bkdA1-bkdA2-bkdB</i>	Branched-chain α -keto acid dehydrogenase E1 (2-oxoisovaleratedehydrogenase α -subunit)

Table 3. (cont.)

Gene	30 min cold shock	60 min cold shock	90 min cold shock	Transcriptional organization	Gene product function (partially putative)
<i>bkdA2</i>	1·7 (0·2)	2·6 (0·3)	3·4 (0·2)	<i>bkdR-ptb-bcd-buk-lpd-bkdA1-bkdA2-bkdB</i>	Branched-chain α -keto acid dehydrogenase E1 (2-oxoisovalerate dehydrogenase β -subunit)
<i>bkdB</i>	2·9 (0·4)	4·8 (1·7)	5·2 (2·9)	<i>bkdR-ptb-bcd-buk-lpd-bkdA1-bkdA2-bkdB</i>	Branched-chain α -keto acid dehydrogenase E2 subunit (lipoamide acyltransferase)
RNA synthesis – elongation					
<i>rpoE</i>	1·6 (0·1)	2·2 (0·2)	2·3 (0·1)	<i>rpoE</i>	RNA polymerase (δ -subunit)
RNA modification					
<i>yugI</i>	1·4 (+) (0·1)	2·8 (+) (0·4)	3·3 (+) (0·5)	<i>yugI</i>	Similar to polyribonucleotide nucleotidyltransferase
Protein synthesis – ribosomal proteins					
<i>rplU</i>	1·2 (0·2)	1·4 (0·1)	1·7 (0·3)	<i>rplU-ysxB-rpmA</i>	Ribosomal protein L21 (BL20)
<i>ysxB</i>	1·2 (0·1)	1·4 (0·1)	1·4 (0·1)	<i>rplU-ysxB-rpmA</i>	Similar to hypothetical proteins
<i>rpmA</i>	1·3 (0·1)	2·4 (0·3)	2·1 (0·2)	<i>rplU-ysxB-rpmA</i>	Ribosomal protein L27 (BL24)
<i>rpmE</i>	1·0 (0·4)	2·1 (0·2)	1·9 (0·2)	<i>rpmE</i>	Ribosomal protein L31
<i>rpsO</i>	1·2 (0·2)	2·4 (0·3)	2·5 (0·3)	<i>rpsO</i>	Ribosomal protein S15 (BS18)
<i>ylbN</i>	1·1 (0·1)	2·2 (0·2)	3·1 (0·2)	<i>ylbN-rpmF</i>	Unknown function
<i>rpmF</i>	0·8 (0·2)	2·3 (0·2)	4·4 (0·8)	<i>ylbN-rpmF</i>	Ribosomal protein L32
Adaptation to atypical conditions					
<i>cspB</i>	2·6 (+) (0·3)	4·6 (+) (1·7)	1·9 (+) (0·1)	<i>cspB</i>	Major cold-shock protein
<i>cspC</i>	1·4 (0·1)	2·1 (0·3)	1·1 (0·2)	<i>cspC</i>	Cold-shock protein
<i>cspD</i>	1·0 (+) (0·1)	1·4 (+) (0·2)	2·9 (+) (0·4)	<i>cspD</i>	Cold-shock protein
Unknown function					
<i>ydeB</i>	1·1 (0·1)	4·9 (2·6)	7·5 (2·8)	<i>ydeB</i>	Similar to proteins of unknown function
<i>ydjO</i>	2·6 (0·2)	4·9 (1·6)	8·1 (3·4)	<i>ydjO</i>	Unknown function
<i>yheA</i>	1·4 (0·1)	2·3 (0·3)	3·3 (1·2)	<i>yheA</i>	Unknown function
<i>ylbF</i>	–	–	–	<i>ylbF-ylbG</i>	Similar to hypothetical proteins
<i>ylbG</i>	1·6 (0·2)	2·1 (0·2)	3·0 (0·4)	<i>ylbF-ylbG</i>	Similar to unknown proteins
<i>yneE</i>	–	–	–	<i>yneE-yneF</i>	Unknown function
<i>yneF</i>	1·5 (0·1)	3·2 (0·7)	3·1 (1·1)	<i>yneE-yneF</i>	Unknown function
<i>yomT</i>	1·0 (0·1)	2·7 (1·4)	2·2 (0·1)	<i>yomT</i>	Unknown function
<i>yopU</i>	1·5 (0·1)	2·4 (0·2)	2·3 (0·1)	<i>yopU</i>	Unknown function
<i>yorD</i>	1·3 (0·1)	3·9 (1·1)	2·9 (0·5)	<i>yorD</i>	Unknown function
<i>ypaA</i>	1·5 (0·2)	3·3 (0·9)	0·9 (0·1)	<i>ypaA</i>	Unknown function
<i>yqfW</i>	2·1 (0·1)	2·9 (0·3)	2·4 (0·1)	<i>yqfW</i>	Unknown function
<i>yqgA</i>	2·4 (0·1)	2·2 (0·1)	4·0 (0·7)	<i>yqgA</i>	Unknown function

Transcriptome analysis: genes exhibiting significantly increased mRNA levels

Approximately 46 genes exhibited significantly increased mRNA levels after the temperature downshift (Table 3). According to the time profile of the mRNA levels, these genes could be assigned to groups of early- and late-induced genes. Most of the induced genes exhibited significantly elevated mRNA levels 60 or 90 min after the temperature downshift. However, a small group of genes revealed significantly increased mRNA levels by 30 min.

The gene encoding the major cold-shock protein CspB belongs to the early cold-shock genes. This protein is assumed to play a crucial role in the adaptational response of *B. subtilis* to low-temperature conditions (Graumann *et al.*, 1997). Further early-induced genes included members of the *ptb-bcd-buk-lpd-bkdA1-bkdA2-bkdB* operon, encoding enzymes involved in the degradation of branched-chain amino acids (Debarbouille *et al.*, 1999), the monocistronic *des* gene, encoding fatty acid desaturase, which forms unsaturated fatty acids from saturated phospholipid precursors (Aguilar *et al.*, 1998), the *mntA-mntB-mntC* operon, encoding a manganese ABC transporter (Que & Helmann, 2000), *yvcE*, encoding a protein similar to cell-wall-binding proteins, the monocistronic *ydjO*, *yqgA* and *yqfW* genes, encoding proteins of unknown function, and the monocistronic *purA* gene, which encodes the adenylosuccinate synthetase involved in AMP biosynthesis (Mantsala & Zalkin, 1992). Interestingly, all of these early-induced genes kept their increased mRNA levels over a time period of at least 90 min after the cold shock. The mRNA levels of *yqgA*, *yvcE*, *ydjO* and the *ptb-bcd-buk-lpd-bkdA1-bkdA2-bkdB* operon revealed a significant further increase within this time. The *des* gene exhibited a 10-fold increase in mRNA level 60 min after the cold shock and thus represents the highest induction ratio of all cold-responsive *B. subtilis* genes identified in this study. After 90 min, a clear decrease of the amount of *des* mRNA was detected, indicating the transient character of *des* up-regulation during cold shock.

The majority of the cold-shock-induced genes was characterized by significantly increased mRNA levels after 60 or 90 min. Two of these genes encode proteins with metabolic functions: the *fbaA* gene, encoding fructose-1,6-bisphosphate aldolase (Tobisch *et al.*, 1999), and the *yrpG* gene, encoding a protein similar to sugar-phosphate dehydrogenase. Among the late-induced cold-shock genes, several exhibited only around twofold increased mRNA levels after 60 as well as 90 min: *rpoE*, encoding the δ -subunit of RNA polymerase, *cspC* and *cspD*, encoding cold-shock proteins, and *yopU*, encoding a protein of unknown function. The operon *yydFGHIJ*, specifying a protein with similarity to an ATP-binding protein of an ABC transporter and four genes encoding products of unknown function, followed the same expression pattern. The late-induced monocistronic genes listed below exhibited a continuous

increase in their mRNA levels up to 90 min after cold shock: *yugI*, encoding a protein similar to polyribonucleotide nucleotidyltransferase, and *yheA* and *ydeB*, encoding proteins of unknown function. The monocistronic genes *yomT*, *yorD* and *ypaA* exhibited a transient increased mRNA pattern with a maximum after 60 min. The late-induced *ylbG* and *yneF* genes most probably represent members of the bicistronic operons *ylbF-ylbG* and *yneE-yneF*, respectively. However, the promoter-proximal genes of these putative operons, *ylbF* and *yneE*, specified signals below the significance threshold. Therefore, it remains unclear if *ylbG* and *yneF* are monocistronically transcribed from promoters immediately upstream of these genes.

Besides the known cold-shock genes *cspB*, *cspC* and *cspD*, there are further candidates, which possibly play roles in the adaptational response of *B. subtilis* cells to low-temperature conditions. In addition to the genes mentioned so far, one group of cold-inducible genes could function in the adaptation of the protein synthesis machinery. After the temperature downshift, we found increased mRNA levels of the following genes encoding ribosomal proteins: *rpmA* (encoding L27), *rpmE* (L31), *rpsO* (S15) and *rpmF* (L32). The *rpmF* gene forms a bicistronic operon together with the promoter-proximal *ylbN* gene, which encodes a protein with unknown function. Both genes exhibited elevated mRNA levels 60 min after the cold shock and a further increase in their mRNA levels after 90 min. The genes *rpmE* and *rpsO* are also co-transcribed as a bicistronic operon. There was a doubling in their mRNA levels 60 min after the temperature downshift and this was still the case after 90 min. Finally, according to the genome sequence, *rpmA* represents the promoter-distal gene of a tricistronic operon *rplU-ysxB-rpmA*. There was a doubling in the *rpmA*-specific mRNA level after 60 and 90 min, whereas the amount of the *rplU-ysxB* transcript was not significantly altered. Possibly, cold-dependent regulation of *rpmA* is mediated by an internal promoter upstream of the gene.

The cold-shock-induced genes identified in this study for which putative functions have been addressed can be affiliated to two main groups. The genes belonging to the first group encode proteins with functions in the adaptation of the cells to low-temperature conditions. The second group encompasses genes encoding proteins required for normal vegetative cellular activities.

The genes *cspB*, *cspC* and *cspD* belong to the first group of cold-shock-induced genes. For *cspB*, the increase in the mRNA level was verified by slot-blot experiments (see below). Furthermore, the up-regulation of *cspB* and *cspD* mRNA levels was also reflected by a strong increase in the amount of the corresponding proteins as revealed by proteome analysis (see below). CspB and its homologous proteins carry conserved motifs that are essential for binding to single-stranded DNA and RNA (Schröder *et al.*, 1995; Lopez *et al.*, 1999, 2001). For CspB and related proteins, an RNA chaperone function has been proposed, facilitating translation at low

temperature by blocking the formation of secondary structures in the mRNA (Graumann & Marahiel, 1999). Recently, Weber *et al.* (2001b) demonstrated that CspB of *B. subtilis* is localized to zones of newly synthesized RNA, most probably coupling transcription with initiation of translation.

The strongest cold-shock-induced gene according to the array analysis was *des*, encoding a desaturase, which forms unsaturated fatty acids from saturated phospholipid precursors, thereby increasing membrane fluidity (Aguilar *et al.*, 1999; Weber *et al.*, 2001a). Membrane fluidity is one of the most important demands for cells during growth at low temperatures to maintain substrate transport via the cell envelope. The strong cold-inducibility of *des* was also verified by slot-blot experiments (see below).

The genes encoding the ribosomal proteins L27, L31, L32 and S15 exhibited increased mRNA levels under cold-shock conditions. It is tempting to speculate that these proteins play a role in the low-temperature adaptation of the *B. subtilis* translational machinery, as suggested for the ribosomal proteins S6 and L7/L12 by Graumann *et al.* (1996).

The second group of cold-shock-induced genes is very diverse. This group includes genes fulfilling functions in the metabolism of carbohydrates (*fbaA*, *yrpG*), nucleotides (*purA*), transport processes (*mntABC*, *yydFGHIJ*), RNA synthesis (*rpoE*) and RNA modification (*yugI*). The *mntABC* operon encodes an ABC transporter for manganese uptake (Que & Helmann, 2000). Transcription of this operon is controlled by the positive transcriptional regulator MntR which is activated in the absence of manganese ions. Possibly, manganese complexes with other ions like phosphates at low temperature and is thus limited. This could in turn activate MntR and consequently induce *mntABC* expression. In this respect it is noteworthy that no increased *mntABC* mRNA levels could be observed after a temperature downshift in Luria Broth (LB) complex medium (data not shown).

In the case of *fbaA*, encoding fructose-1,6-bisphosphate aldolase, there was a significant increase in the mRNA amount after 90 min cold shock. Interestingly, the proteome approach revealed a significant decrease in the FbaA protein level 30, 60 and 90 min after the temperature downshift. A possible explanation could be that the *fbaA*-specific mRNA is translationally blocked under cold-shock conditions, causing a reduction in the amount of the encoded protein. The late increase in the RNA level would then indicate the cellular reaction to equalize the lack in fructose-1,6-bisphosphate aldolase. A similar mechanism might be responsible for the increase in *purA* mRNA. This gene encodes the adenylosuccinate synthetase involved in purine biosynthesis and belongs to the genes with the highest increased mRNA levels under the conditions tested in this study. On the other hand, as in the case of *fbaA*, the proteome approach revealed a decreased level of the corresponding PurA protein after 30, 60 and 90 min cold shock.

The cold-shock-dependent regulation of the heptacistronic operon *ptb-bcd-buk-lpd-bkdA1-bkdA2-bkdB* is of special interest. The enzymes encoded by this operon catalyse the degradation of the branched-chain amino acids isoleucine and valine to branched-chain carboxylic acids (Debarbouille *et al.*, 1999). Transcription of this operon has been shown to be strongly dependent on the alternate sigma factor SigL and positively regulated by the regulator BkdR. Furthermore, there is also a negative regulation via the transcriptional repressor CodY. The degradation intermediates of isoleucine and valine, α -methylbutyryl-CoA and isobutyryl-CoA, are utilized for synthesis of anteiso-branched fatty acids. These fatty acids, as components of membrane lipids, contribute essentially to sufficient fluidity of the membrane under low-temperature conditions. At low temperature, the content of anteiso-branched fatty acids in *B. subtilis* increases and the level of iso-branched fatty acid species decreases (Klein *et al.*, 1999). Besides the activity of the fatty acid desaturase, this system also contributes to the reorganization of the cell membrane at low temperature. However, the mechanism mediating the increase of the mRNA level of the *ptb-bcd-buk-lpd-bkdA1-bkdA2-bkdB* operon under cold-shock conditions remains unclear.

The increased mRNA levels identified by the transcriptome analyses in this study could be due to either an indirect post-transcriptional effect, such as the strong stabilization of the *cspB* or *cspC* mRNAs (Kaan *et al.*, 1999) or a direct positive regulation at the transcriptional level. Recently, a two-component signal transduction system has been determined, which positively regulates the expression of the *des* gene, encoding the desaturase of *B. subtilis* under low-temperature conditions (Aguilar *et al.*, 2001). These authors suggested that the DesK/DesR two-component system functions like a cold-shock thermometer. It could be demonstrated by footprinting experiments that the response regulator DesR binds to a specific region upstream of the *des* promoter. To verify whether additional cold-shock-induced genes could be regulated by this two-component system, we performed a sequence pattern search with the suggested binding region of DesR. However, none of the cold-shock-induced genes identified in this study was preceded by a similar sequence motif.

Verification of DNA macroarray results by RNA slot-blot analysis

To check the significance of the DNA array data, follow-up experiments were carried out. First, the RNA levels of selected cold-inducible genes derived from the transcriptome study were analysed by slot-blot experiments (Fig. 1). Altogether seven genes, all exhibiting significantly increased mRNA levels under cold-shock conditions, were chosen: *mntA*, *purA*, *yydG*, *yvcE*, *des*, *cspB* and *cspC*. The mRNA level of the first gene of the *mntABC* operon, *mntA*, was maximally increased 2.5-fold in the array experiment after 60 min. The slot-blot analysis revealed a corresponding maximal induction factor of 3.5 after 60 min. The *purA* gene, showing a

Fig. 1. Slot-blot analysis of mRNA levels of *apt*, *yvfG*, *yydG*, *yvcE*, *purA*, *mntA*, *des*, *cspB* and *cspC* at 37 °C (C), and 30, 60 and 90 min after the temperature downshift to 18 °C. The relative mRNA level is indicated on the right of each band.

maximally fivefold increased mRNA level after 60 min, according to the DNA array, was increased about 20-fold at the same time point in the slot-blot experiment and reached a maximal mRNA level after 90 min (about 40-fold). This represented the highest mRNA level ratio of all cold-shock-sensitive mRNAs checked by slot-blot analysis and the largest discrepancy between global (DNA macroarray) and detailed (slot-blot) approaches. The *yydG* gene represents the second gene of the pentacistronic *yydFGHIJ* operon, whose mRNA level was increased about twofold after 60 and 90 min in the

array experiment. The slot-blot analysis gave comparable mRNA ratios of 1.9 and 3.2. A similarly good agreement was found in the case of the monocistronic *yvcE* gene: the array analysis revealed mRNA ratios of 2.7, 4.9 and 4.6 after 30, 60 and 90 min cold stress; the respective slot-blot values were 2.1, 2.2 and 4.1. The *des* gene encoding desaturase exhibited the strongest induction in the array experiments: a 2.5-fold increase in *des*-specific mRNA after 30 min was followed by the peak of induction (around 10-fold) after 60 min and a subsequent decrease in the mRNA level to a ratio of 3.3

after 90 min. This transient induction pattern was also reflected in the slot-blot experiments, although the absolute values for the induction ratios were higher: 12.1, 18.2 and 15.8, respectively. A transient increase in the mRNA level was also verified in the case of the *cspB* gene encoding the major cold-shock protein CspB. The array analysis resulted in mRNA ratios of 2.6 (30 min), 4.6 (60 min) and 1.9 (90 min), corresponding to ratios of 5.3, 3.8 and 1.5 in the slot-blot experiment. In the case of the *cspC* mRNA, the macroarray analysis indicated a transient increase after 60 min. The slot-blot experiments revealed an increase by 30 min, followed by similar high mRNA levels after 60 and 90 min.

In general, the results of the global transcriptome approach and the detailed analysis are comparable. Genes with significantly increased mRNA levels in the transcriptome study also showed increased mRNA levels in the slot-blot approach. In most cases, even the extent of the induction was similar. Therefore, the transcriptome data were broadly confirmed by the slot-blot experiments.

Comparison of DNA macroarray results with proteome data

The DNA array results were checked further by a corresponding proteome analysis using a 2D PAGE technique. Comparison of [³⁵S]methionine-labelled cytoplasmic protein fractions prepared from cells cultivated at 37 °C and at different times after the temperature downshift demonstrated that the protein synthesis capacity of the cold-shocked cells is drastically decreased. The incorporation rate of [³⁵S]methionine was reduced by a factor of five after the temperature downshift from 37 to 18 °C. Interestingly, the transcriptome approach resulted in the identification of only 52 genes exhibiting decreased mRNA levels, whereas the proteome approach revealed the down-regulation of the synthesis of significantly more proteins: about 30% of the protein spots visible in the control gel were not detectable in the 2D protein gels of the cold-shocked cells. This discrepancy verifies the earlier idea that the strong global down-regulation of gene expression under cold-shock conditions results primarily from inhibition of translation due to stabilized secondary structures of the mRNA. Among the proteins whose synthesis was strongly reduced after cold shock were IlvB, LeuA, LeuB, LeuC and IlvD, all identified as down-regulated by the transcriptomics approach, as shown in Table 2. The decreased mRNA levels of *guaB*, *purF*, *thiA*, *ykrT*, *ykrS* and *yjbC* were also reflected at the protein level (Table 2). In all cases, apart from IlvC, the down-regulated genes also showed a decreased expression at the protein level, if detectable on the 2D gels. In the macroarray analysis, all genes of the *ilvBHClcuABCD* operon revealed decreased mRNA levels, but the gene product of *ilvC* increased at the protein level.

About 43 proteins demonstrating increased levels after the temperature downshift were identified by means of the 2D PAGE approach. The most dominant protein

spots after the temperature downshift were represented by the cold-shock proteins CspB and CspD. The increased amount of these cold-shock proteins was observed 30 min after the temperature downshift. In the case of CspB, this expression pattern corresponds to the transcriptome analysis (Table 3), whereas in the case of CspD, an increased mRNA level was only detectable after 90 min. Furthermore, the increased mRNA level of the *yugI* gene, encoding a putative polyribonucleotide nucleotidyltransferase, was also verified by the proteomics approach. In contrast, the proteins PurA and FbaA were present in lower amounts under cold-shock conditions, although their mRNA levels were increased.

Besides the cold-shock proteins CspB and CspD, the ribosomal proteins S6 and L7/L12 belonged to the most highly accumulating proteins under cold-shock conditions in *B. subtilis*. The number of cold-shock-induced proteins is pretty consistent with results from Graumann *et al.* (1996) who identified 37 cold-shock proteins by the 2D PAGE approach. However, there are slight differences between these two studies. We did not find significantly increased protein levels of the prolyl-isomerases PpiB and Tig, the cysteine synthase CysK or the glyceraldehyde phosphate dehydrogenase Gap under the conditions examined in this study. Although there are slight differences in the experimental design of Graumann *et al.* (1996) in comparison to our study, the reasons for these differences are not clear.

A considerable part of the proteins encoded by genes showing significantly increased mRNA levels in the DNA array experiments was not visible in the 2D PAGE analyses. In most cases, this was due to either an unfavourable alkaline pH or a low protein level.

Whereas the mRNA levels of most of the cold-shock-induced genes reached their maximum 60 min after the temperature downshift, the level of the majority of the cold-shock proteins was highest after 90 min. This is consistent with the data of Graumann *et al.* (1996) who demonstrated that the cold-shock response in *B. subtilis* indeed represents a transient regulated phenomenon.

Growth experiments of strains carrying null mutations in cold-shock-induced genes

The mutant strains BFS4469, BFS4564, YHEAd and BFS4794 carry pMUTIN integrations in the genes *ydeB*, *ydjO*, *yheA* and *yqgA*, which were identified as strongly cold-shock-induced in the transcriptome analysis. These mutants were analysed for a cold-shock-sensitive growth phenotype.

Strains were inoculated with an exponentially growing preculture to an initial OD₅₀₀ of 0.04 and cultivated at 37 °C until reaching an OD₅₀₀ of 0.3. Cultures were then shifted to 15 °C by transferring them to a precooled shaker incubator and incubated for 30 h. Two different minimal media were used (see Methods). The experiments were carried out several times and generated highly reproducible results. All mutants revealed a weak but significant growth disadvantage in comparison to

the wild-type strain *B. subtilis* 168. These decreased growth rates were most probably due to the necessity to cultivate the mutant strains in the presence of erythromycin and lincomycin to stabilize the single-crossover integration of the pMUTIN. Therefore, BFS4226, carrying a pMUTIN integration in *yacK* was used as an additional control. This strain was chosen because its expression was not changed by the cold shock. The *yacK* mutant showed the same growth behaviour as the other mutant strains (data not shown). Consequently, it has to be concluded that, at least under the conditions tested in this study, inactivation of the cold-inducible genes *ydeB*, *ydjO*, *yheA* and *yqgA* does not significantly impair the fitness of *B. subtilis* at low temperature.

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