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# Adaptation of *Bacillus subtilis* to growth at low temperature: a combined transcriptomic and proteomic appraisal

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The soil bacterium *Bacillus subtilis* frequently encounters a reduction in temperature in its natural habitats. Here, a combined transcriptomic and proteomic approach has been used to analyse the adaptational responses of *B. subtilis* to low temperature. Propagation of *B. subtilis* in minimal medium at 15 °C triggered the induction of 279 genes and the repression of 301 genes in comparison to cells grown at 37 °C. The analysis thus revealed profound adjustments in the overall gene expression profile in chill-adapted cells. Important transcriptional changes in low-temperature-grown cells comprise the induction of the SigB-controlled general stress regulon, the induction of parts of the early sporulation regulons (SigF, SigE and SigG) and the induction of a regulatory circuit (RapA/PhrA and Opp) that is involved in the fine-tuning of the phosphorylation status of the Spo0A response regulator. The analysis of chill-stress-repressed genes revealed reductions in major catabolic (glycolysis, oxidative phosphorylation, ATP synthesis) and anabolic routes (biosynthesis of purines, pyrimidines, haem and fatty acids) that likely reflect the slower growth rates at low temperature. Low-temperature repression of part of the SigW regulon and of many genes with predicted functions in chemotaxis and motility was also noted. The proteome analysis of chill-adapted cells indicates a major contribution of post-transcriptional regulation phenomena in adaptation to low temperature. Comparative analysis of the previously reported transcriptional responses of cold-shocked *B. subtilis* cells with this data revealed that cold shock and growth in the cold constitute physiologically distinct phases of the adaptation of *B. subtilis* to low temperature.

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## INTRODUCTION

A primary habitat of the Gram-positive bacterium *Bacillus subtilis* is the upper layer of the soil. Within this ecosystem, *B. subtilis* experiences a wide variety of environmental challenges and nutrient limitations (Wipat & Harwood, 1999) that in extreme cases can induce the formation of a highly resistant endospore (Sonenshein, 2000). Changes in temperature constitute a key factor that influences cell growth and survival in the soil. Under laboratory conditions, *B. subtilis* is able to sustain growth in a temperature range from approximately 11 °C (Nichols *et al.*, 1995) to 52 °C (Holtmann & Bremer, 2004). Adaptation responses of the cell are triggered when the temperature is rapidly increased or decreased within this temperature range (Hecker *et al.*, 1996; Weber & Marahiel, 2002), or when the cells are cultivated for prolonged periods close to the upper or lower temperature limits (Brigulla *et al.*, 2003; Holtmann *et al.*, 2004).

The changes in gene expression that occur following a rapid increase in temperature are collectively known as the heat-shock response (Hecker *et al.*, 1996; Schumann *et al.*, 2002). Heat-shock proteins help the cell to refold temperature-damaged proteins via chaperones and to degrade thermally denatured polypeptides via proteases (Schumann *et al.*, 2002). A sudden increase in growth temperature also triggers a rapid induction of a large general stress regulon (Hecker & Völker, 2001; Price, 2002) that is controlled by

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Abbreviations: ABC, ATP-binding cassette; 2-DE, two-dimensional protein gel electrophoresis; 2D-DIGE, two-dimensional differential in-gel protein electrophoresis.

Supplementary material is available with the online version of this paper.

the alternative transcription factor SigB (Benson & Haldenwang, 1993; Völker *et al.*, 1994). Induction of the SigB-dependent general stress regulon provides *B. subtilis* cells with a multiple, non-specific and pre-emptive stress resistance that includes protection against a growth-inhibiting heat shock (54 °C) (Hecker & Völker, 2001; Höper *et al.*, 2005; Völker *et al.*, 1999).

Heat-shock proteins such as GroEL, GroES, DnaK and GrpE are also detected at an increased level when *B. subtilis* is propagated close to its upper temperature limit (Holtmann *et al.*, 2004). This growth condition also triggers a sustained high-level expression of the entire general stress regulon (Holtmann *et al.*, 2004). Effective thermoprotection of *B. subtilis* can also be accomplished by the uptake of exogenously provided compatible solutes, such as glycine betaine and the amino acid glutamate (Holtmann & Bremer, 2004).

A sudden drop from the standard growth temperature (37 °C) down to 15 °C is generally used in the laboratory to elicit the cold-shock response of *B. subtilis* (Graumann & Marahiel, 1996; Weber & Marahiel, 2002). Such a cold shock imposes constraints on the *B. subtilis* cell that are different from those triggered by heat shock. A rapid decrease in temperature hampers the translational capacity of the cell due to the formation of increasingly stable mRNA secondary structures and diminished ribosome activity, results in slower protein folding and reduced protein activity, and impairs the fluidity of the cytoplasmic membrane, thereby negatively affecting transport processes (Mansilla *et al.*, 2004; Weber & Marahiel, 2002). *B. subtilis* mounts different sets of cellular defence reactions to cope with these constraints.

One hallmark of the cold-shock response of *B. subtilis* is the high-level production of so-called cold-shock proteins (CspB, CspC, CspD) (Graumann *et al.*, 1996). These proteins act as RNA chaperones that improve the protein-synthesis capacity of the cell by specifically binding to single-stranded nucleic acids, thereby probably reducing the formation of mRNA secondary structures. They are essential for effective protein synthesis and cell viability at both low and optimal growth temperatures (Graumann *et al.*, 1997). Double *csp* deletion strains display multiple phenotypes, such as cell lysis upon entry into the stationary growth phase, aberrant nucleoid structure, and impairment in sporulation (Graumann *et al.*, 1997; Weber *et al.*, 2001b).

Rigidification of the cytoplasmic membrane at low temperature can be prevented in *B. subtilis* by either the isoleucine-dependent *de novo* synthesis of branched-chain fatty acids (Klein *et al.*, 1999) or the desaturation of pre-existing fatty acids by a cold-shock-induced, membrane-bound phospholipid desaturase (Des) (Aguilar *et al.*, 1998; Weber *et al.*, 2001a). Both pathways result in reduced attraction between adjacent fatty acid chains, thereby appropriately adjusting membrane fluidity to the low-temperature environment (Mansilla *et al.*, 2004). Expression of the gene (*des*) encoding the phospholipid desaturase is controlled by a two-component regulatory system, DesK/DesR (Aguilar *et al.*, 2001). The membrane-embedded sensor kinase DesK monitors membrane fluidity and controls, via the phosphorylation of its cognate response regulator DesR, the transient induction of the structural gene for the Δ5-lipid desaturase upon a temperature downshift (Aguilar *et al.*, 1999, 2001). This two-component system seems to exclusively control transcription of the *des* gene (Beckering *et al.*, 2002), and hence does not function as a general cold-shock sensory and regulatory device for *B. subtilis*. Recent genome-wide transcriptional profiling studies of cold-shocked *B. subtilis* cells have revealed many additional changes in gene expression, but the impact of these changes on cold adaptation is largely unexplored (Beckering *et al.*, 2002; Kaan *et al.*, 2002).

The drastic and rapid temperature downshifts used under laboratory conditions to provoke a cold-shock response in *B. subtilis* are unlikely to occur frequently in nature. In the upper layers of the soil, gradual changes in temperature prevail, and the cells have to adapt to and continue to grow under chill-stress conditions. This facet of low-temperature adaptation by *B. subtilis* is less well characterized than the cold-shock response. A proteomic study by Brigulla *et al.* (2003) of cells growing at low temperature (15 °C) revealed a delayed but sustained induction of the SigB-dependent general stress regulon. Mutations in the structural gene for the central regulator SigB profoundly affect growth under chill-stress conditions (Brigulla *et al.*, 2003) and also reduce stationary-phase survival of cold-adapted cells (Mendez *et al.*, 2004). Sensitization against growth at low temperature is also observed in a set of mutants lacking selected general stress proteins (Höper *et al.*, 2005). Furthermore, the compatible solute glycine betaine not only provides protection against high growth temperature (Holtmann & Bremer, 2004) but also greatly improves growth of *B. subtilis* under chill-stress conditions, and can even rescue the growth defect of a *sigB* mutant at 15 °C (Brigulla *et al.*, 2003). An additional aspect of chill adaptation by *B. subtilis* has recently been discovered by Mendez *et al.* (2004), who have detected a novel role for the Spo0A response regulator in cellular viability and stress survival at low temperature that is independent of its central role as the master switch in the initiation of sporulation (Hoch, 1995).

To foster our understanding of the adaptational response of the cell to continued growth at low temperature, we used a combined transcriptomic and proteomic approach to evaluate the changes that occur in *B. subtilis* cells cultivated at low temperature, so as to define the chill-stress stimulon on a global scale. Transcriptional analysis revealed that major physiological changes occur in the *B. subtilis* cell when it is actively growing under chill-stress conditions, affecting approximately 14 % of all *B. subtilis* genes. Our proteome analysis of chill-adapted cells suggests a major contribution of post-transcriptional regulation phenomena in the adaptation reaction of *B. subtilis* to growth at low temperature.

## METHODS

**Bacterial strains and strain construction.** The experiments conducted in this study were performed with the *B. subtilis* wild-type strain 168 (*trpC2*) (Kunst *et al.*, 1997) and several mutant derivatives. Mutations were transferred into the *B. subtilis* 168 strain background by transformation (Harwood & Cutting, 1990). We used two different *sigB* mutant derivatives of strain 168 in this study. Strain BSM29 (Brigulla *et al.*, 2003) carries the *sigBΔ2::spc* allele, and strain BSM27 was constructed by transferring the *sigB*::Δ*Hin*dIII-*Eco*RV::*cat* allele from strain ML6 (Igo *et al.*, 1987) into the 168 background and by selecting chloramphenicol-resistant transformants. The *sigF* (Δ*spoIIA*) mutant was constructed by transforming strain 168 with chromosomal DNA isolated from strain BSG03 (Δ*spoIIA*::*spc amyE*::pGK30-*gsiB*::*gfp cat86*; U. Völker, unpublished data) and selecting for spectinomycin-resistant transformants, thereby obtaining strain IBB1. The *sigB sigF* double mutant IBB2 was generated by transforming strain BSM27 with chromosomal DNA from strain IBB1 and screening for colonies that simultaneously exhibited resistance to chloramphenicol and spectinomycin. The *sigB sigF sigG* triple-mutant strain BSG27 was constructed in two steps. First, competent cells of the *sigB* mutant strain BSM27 were transformed with chromosomal DNA of the *sigG* mutant strain Marb04 (Steil *et al.*, 2005). Selection for spectinomycin-resistant colonies yielded strain BSG26 (*sigB*::Δ*Hin*dIII-*Eco*RV::*cat spoIIIG*::*spc*). In a second step, competent cells of this *sigB sigG* double mutant were transformed with chromosomal DNA of strain MO1073 (*spoIIAC*::*erm*) (Stragier *et al.*, 1988), and erythromycin-resistant colonies were selected. This yielded strain BSG27. The presence of the resistance cassettes in the structural genes for the different sigma factors was verified by PCR. For drug-resistance selection in *B. subtilis*, antibiotics were used at the following final concentrations: chloramphenicol, 5 μg $ml^{-1}$; spectinomycin, 200 μg $ml^{-1}$; and erythromycin, 1 μg $ml^{-1}$.

**Media and growth conditions.** *B. subtilis* strains were maintained on Luria–Bertani (LB) medium containing appropriate antibiotics; plates were incubated at 37 °C. Bacteria were routinely grown with vigorous agitation (220 r.p.m.) in an aerial shaker in Spizizen's Minimal Medium (SMM) with 0·5 % (w/v) glucose as the carbon source, L-tryptophan (20 mg $l^{-1}$) and a solution of trace elements (Harwood & Archibald, 1990). Pre-cultures of *B. subtilis* strains were inoculated from exponentially growing overnight cultures that had been propagated in SMM to a final $OD_{578}$ of 0·1. For the cultures exclusively propagated at 37 °C, cells were harvested after mixing the samples with an equal volume of frozen killing buffer (20 mM $NaN_3$, 20 mM Tris/HCl, pH 7·5, 5 mM $MgCl_2$) by centrifugation (10 min at 6000 ***g*** at 4 °C) when the cultures had reached an $OD_{578}$ of 1·0. These cells were used either for the isolation of total RNA for DNA array experiments or for the isolation of crude protein extracts for proteome studies. For the growth of *B. subtilis* at low temperature, pre-cultures were propagated to an $OD_{578}$ of 0·5 at 37 °C, diluted to an $OD_{578}$ of 0·1 and subsequently transferred to the lower growth temperatures (15 or 16 °C) indicated in the individual experiments. The growth regimen applied allowed growth of the cultures up to an $OD_{578}$ of at least 6, and cultures were propagated at the lower temperature for at least three generations before harvesting. At 72 ± 8 h after the transfer to the lower temperature, when the $OD_{578}$ had reached 1·0, aliquots of the cultures were harvested by mixing the samples with an equal volume of frozen killing buffer and by subsequent centrifugation for 10 min at 6000 ***g*** and 4 °C. The pellets were stored at −80 °C until used for RNA or protein preparation.

**Cell lysis and RNA isolation.** Total RNA of *B. subtilis* was isolated after mechanical disruption of frozen cells in a Micro-Dismembrator (B. Braun Biotec Int., Melsungen, Germany) as described by Hauser *et al.* (1998), using the modifications introduced by Petersohn *et al.* (2001).

**Northern blot analysis.** Aliquots of the total RNA prepared for the DNA macroarray experiments were used for Northern blot analysis of the transcription of individual *B. subtilis* genes. RNA electrophoresis on agarose gels, RNA transfer by diffusion onto a Nylon membrane (NY13N; Schleicher & Schuell), hybridization to gene-specific probes, and signal detection were performed as described by Holtmann *et al.* (2003). Digoxigenin (DIG)-labelled anti-sense RNA probes were generated by *in vitro* transcription using a StripEZ-kit (Ambion, Inc.) and gene-specific PCR products as templates. In each of the PCR reactions with chromosomal DNA prepared from *B. subtilis* strain 168, one of the DNA primers carried the sequence of the T7 promoter. The PCR fragment was subsequently used for *in vitro* RNA synthesis with commercially available T7 RNA polymerase (Ambion, Inc.). These reactions yielded hybridization probes internal to the structural genes of the following size: *spo0A* (621 nt), *spoIIIG* (272 nt), *sspE* (229 nt), *yhcM* (429 nt), *bofC* (455 nt), *spoIVA* (602 nt), *cotE* (475 nt), *yjbX* (638 nt), *spoIIID* (272 nt), *rapA* (609 nt), *phrA* (118 nt), *yoaA* (465 nt), *yoxB* (539 nt), *yoxC* (272 nt), *ywcI* (283 nt), *sacT* (548 nt), *dps* (351 nt), *oppA* (665 nt), *oppF* (661 nt), *wapA* (450 nt), *yxxG* (331 nt), *htrA* (501 nt), *ldh* (505 nt), *lctP* (525 nt), *spo0M* (682 nt) and *fabHB* (713 nt). The nucleotide sequences of the DNA primers used for the generation of the PCR products are available upon request.

**Preparation of labelled cDNA, array hybridization and DNA macroarray regeneration.** Prior to the cDNA labelling, the overall integrity of the total RNA preparation was analysed using a Bioanalyser 2100 capillary electrophoresis system (Agilent Technologies). cDNA synthesis and labelling were performed exactly as described by Steil *et al.* (2003). Transcriptional profiling was carried out with commercially available Panorama *B. subtilis* DNA macroarrays (Sigma Genosys Ltd) that carry duplicate spots of PCR products representing 4107 *B. subtilis* genes. cDNA denaturation, probe hybridization and washing of the filters were performed as described by Petersohn *et al.* (2001), with the modifications detailed by Steil *et al.* (2003). Hybridized arrays were then exposed to storage phosphor screens (Molecular Dynamics) for 2 to 4 days, and subsequently scanned with a Storm 840/860 phosphorimager (Molecular Dynamics) at a resolution of 50 μm and a colour depth of 16 bit. Bound cDNA was stripped off the DNA macroarray membranes by three washing cycles involving a short (1 min) washing step with 250 ml of boiling buffer (5 mM sodium phosphate, pH 7·5, 0·1 % SDS) and an incubation in 250 ml of fresh buffer at 95 °C for 20 min.

**Data analysis of the transcriptional profiling experiments.** Data analysis followed a three-step procedure. First, the ArrayVision software (Version 6.1, Imaging Research, St Catherines, ON, Canada) was used for the quantification of the hybridization signals after direct import of the phosphorimager files. The analysis yielded the artefact-removed volumes (ARVol) and background values, calculated from the median of a line surrounding each group of eight spots on the array. These data were then used in a second step in Microsoft Excel to calculate for every spot on the array a quality score that reflected the ratio between the signal intensity and the background intensity (see supplementary material). This quality score was utilized to identify the hybridization signals close to the detection limit, thereby avoiding artificially high induction ratios for those genes. Data normalization and data analysis were done in a third step with GeneSpring version 7.2 (Agilent Technologies). Gene expression was considered to be changed when three criteria were fulfilled: (i) expression of the gene had to exceed the background signal level by a threshold determined as described in the supplementary material; (ii) changes in expression of the gene had to be statistically significant, as defined in a statistical group comparison of the values of the selected conditions with a parametric test (Welch *t* test; *P* value cut-off 0·01) and a Benjamini and Hochberg false discovery rate correction with a *P* value cut-off of 0·01, as

defined in the GeneSpring software package; and (iii) the change in expression had to exceed a factor of two. Calculations of ratios were done with means of the four parallel samples described below. For transcriptional profiling, mRNA was prepared from four independent cultivations of *B. subtilis* cells and then used for independent cDNA synthesis and DNA array hybridizations. The Panorama *B. subtilis* DNA macroarrays contained duplicated DNA samples for each of the 4107 *B. subtilis* genes, therefore the processing of four independent samples for each growth condition yielded eight data points for the calculation of signal intensities for each gene. The potential functions of the encoded proteins were inferred from the SubtiList database (http://genolist.pasteur.fr/SubtiList/) (Moszer *et al.*, 2002).

**Two-dimensional differential in-gel protein electrophoresis (2D-DIGE).** Cell pellets were resuspended in Tris/EDTA buffer (10 mM Tris/HCl, pH 7·5, 1 mM EDTA) and bacteria were disrupted by sonication. After removal of cell debris by centrifugation (20 000 ***g*** for 30 min at 4 °C), the protein concentration of the crude protein extracts was determined according to the method of Bradford (1976). Prior to the two-dimensional protein gel electrophoresis (2-DE), protein extracts were labelled with Cy-dyes according to the manufacturer's instructions (GE Healthcare, Freiburg, Germany). Briefly, after adjustment of the pH of the protein extract to 8·5 with 50 mM NaOH, 150 μg of each protein extract was labelled with 1200 pmol (400 pmol $\mu l^{-1}$) of Cy5 or Cy3 dye. Furthermore, aliquots (75 μg) of each of the four samples [wild-type strain 168 grown at 37 °C, wild-type strain 168 grown at 16 °C, strain BSM29 (*sigB*) grown at 37 °C and strain BSM29 (*sigB*) grown at 16 °C] were mixed and labelled with 2400 pmol (400 pmol $\mu l^{-1}$) of Cy2 dye, generating a pooled standard of all proteins present in the crude protein extracts. Labelling of 150 μg of protein extract from each experimental condition allowed the separation of three replicates by 2-DE. 2D-DIGE gels were loaded with 50 μg of the Cy2-labelled pooled standard, a Cy5-labelled sample and a Cy3-labelled sample. In order to allow direct comparison of protein patterns of wild-type cells of strain 168 grown at 37 °C (Cy5 labelled) and 16 °C (Cy3 labelled), the corresponding protein extracts were separated together with the pooled standard. An additional set of three gels was used to separate protein extracts of cells of the *sigB* mutant strain BSM29 grown at 37 °C (Cy5 labelled) or 16 °C (Cy3 labelled) together with the pooled standard. Mixed Cy-dye-labelled protein extract (150 μg) was added to a rehydration solution containing 8 M urea, 2 M thiourea, 2 % (w/v) CHAPS, 28 mM DTT, 1·3 % (v/v) Pharmalytes, pH 3–10, and bromphenol blue. Immobilized pH gradient (IPG) strips (GE Healthcare) with a pH range from 4 to 7 were rehydrated in the protein-containing solution for 24 h under low-viscosity paraffin oil. Subsequently, the IPG strips were subjected to isoelectric focusing with the following voltage/time profile: a linear increase from 0 to 500 V for 1000 Vh, 500 V for 2000 Vh, a linear increase from 500 to 3500 V for 10 000 Vh, and a final phase of 3500 V for 35 000 Vh. IPG strips were incubated consecutively for 15 min each in equilibration solutions A and B [solution A: 50 mM Tris/HCl, pH 6·8, 6 M urea, 30 % (v/v) glycerol, 4 % (w/v) SDS, 3·5 mg DTT $ml^{-1}$; solution B, as solution A, but 45 mg iodoacetamide $ml^{-1}$ instead of DTT]. In the second dimension, proteins were separated on 12·5 % SDS-PAGE gels with the Dodecan Electrophoresis System (Bio-Rad) at 2 Watts per gel. After each gel had been scanned with separate laser excitation for each of the three Cy-dyes with a Typhoon scanner (GE Healthcare), analysis of the 2-DE images was performed with version 3.3 of the Delta2D software package (Decodon GmbH, Greifswald, Germany). All gel images (three dyes by six gels) were matched with the Delta2D software and fused to a master gel; subsequently, spot detection was performed for this fusion gel. The spot mask of the fusion gel was then transferred to each of the three individual 2-DE images (Cy2, Cy3, Cy5) of the six gels. After background subtraction, spot volumes were calculated with the Delta2D software and normalized both to the corresponding image (relative expression of each spot compared to the total spot volume of the image) and the pooled Cy2 standard of each individual spot (ratio of the particular sample spot volume to the Cy2 spot volume of the internal standard). The resulting values, which were normalized for image and pooled standard (percentage volume as defined by the Delta2D software) were exported to the GeneSpring software version 7.2 (Agilent Technologies). Protein levels were considered to be changed when two criteria were fulfilled: (i) changes in the level of the protein had to be statistically significant, as defined in a statistical group comparison of the values of the selected conditions with a parametric test (Welch *t* test, *P* value cut-off 0·05), as defined in the GeneSpring software package; and (ii) the change in level had to exceed a factor of two. Calculations of ratios were done with averages of the three parallel samples described above.

**Preparation of peptide mixtures for matrix-assisted laser desorption ionization (MALDI)-MS.** After the imaging with the Typhoon imager, the 2-DE gels were stained with colloidal Coomassie brilliant blue. After matching spot patterns of gels stained with Coomassie brilliant blue and Cy-dye, proteins were excised from stained 2-DE gels using a spot cutter (Proteome Works, Bio-Rad) with a picker head of 1·5 mm diameter, and transferred into 96-well microplates loaded with 100 μl Lichrosolv water per well. Digestion with trypsin and subsequent spotting of peptide solutions onto the MALDI targets were performed automatically in the Ettan spot handling workstation (GE Healthcare) using a modified standard protocol that has been described elsewhere (Eymann *et al.*, 2004). The matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) measurement of spotted peptide solutions was carried out on a 4700 Proteomics Analyser (Applied Biosystems), as described by Eymann *et al.* (2004). MS/MS analysis was performed for the three strongest peaks of the MS spectrum. For one main spectrum, 20 subspectra with 125 shots per subspectrum were accumulated using a random search pattern. The internal calibration was automatically performed as one-point calibration if the monoisotopic arginine $(M+H)^+$ $m/z$ at 175·119 or lysine $(M+H)^+$ $m/z$ at 147·107 reached a signal-to-noise (S/N) ratio of at least 5. The peak lists were created using the 'peak to mascot' script of the 4700 Explorer software (Applied Biosystems) with the following settings: mass range from 60 Da to a mass that was 20 Da lower than the precursor mass; peak density of 10 peaks per 200 Da; minimal area of 100 and maximal 100 peaks per precursor; minimal S/N ratio of 5. Database searches employed a *B. subtilis*-specific database using the Mascot search engine (Matrix Science Ltd, London, UK). The identification of a protein spot was considered significant if the Mowse score exceeded a value of 49, which corresponds to a *P* value of 0·05. Potential protein functions were inferred from the SubtiList database (http://genolist.pasteur.fr/SubtiList/) (Moszer *et al.*, 2002).

## RESULTS AND DISCUSSION

### Chill-induced changes in the transcriptional profile of *B. subtilis* cells

To monitor differences in the transcriptional profile between cells continuously growing at 15 °C versus cells grown at 37 °C, we propagated four independent cultures of the wild-type strain 168 (*trpC2*) in a chemically defined minimal medium (SMM) with glucose as the carbon source. Total RNA was prepared from exponentially growing cultures ($OD_{578}$ of 1) and used for the preparation of radiolabelled cDNA, which was then hybridized to commercially available Panorama *B. subtilis* DNA macroarrays that represent 4107

protein-encoding genes from *B. subtilis* (Kunst *et al.*, 1997). The expression pattern of each of the cultures grown in parallel was highly reproducible, and on average showed Pearson correlation coefficients of 0·972 (data not shown). In contrast, the expression pattern of samples grown at 15 °C differed significantly from those of cultures grown at 37 °C (Pearson correlation coefficients of 0·861). A group of 279 genes displayed at least a twofold statistically significant induction in cultures grown at 15 °C, and 301 genes displayed at least a twofold statistically significant repression under chill-stress growth conditions (Fig. 1A). The 279 genes whose transcription is induced in the cold are listed in Table 1, together with their predicted function as currently annotated in the SubtiList database (Moszer *et al.*, 2002). We also list in this table known or suggested regulatory proteins for these genes. Detailed information on the 301 genes whose activity is reduced at low growth temperature is available in Supplementary Table 2.

## Validation of transcriptional profiling results by Northern blot analysis

To assess the reliability of the transcriptional profiling data, we chose three transcriptional units that were induced under chill stress and five transcriptional units that were repressed in cells grown at low temperature. We then carried out a Northern blot analysis. The data summarized in Fig. 2 demonstrate that in each case the pattern observed in the DNA array analysis was confirmed in the Northern blot analysis, thereby supporting our identification of differentially expressed genes via the DNA array technique.

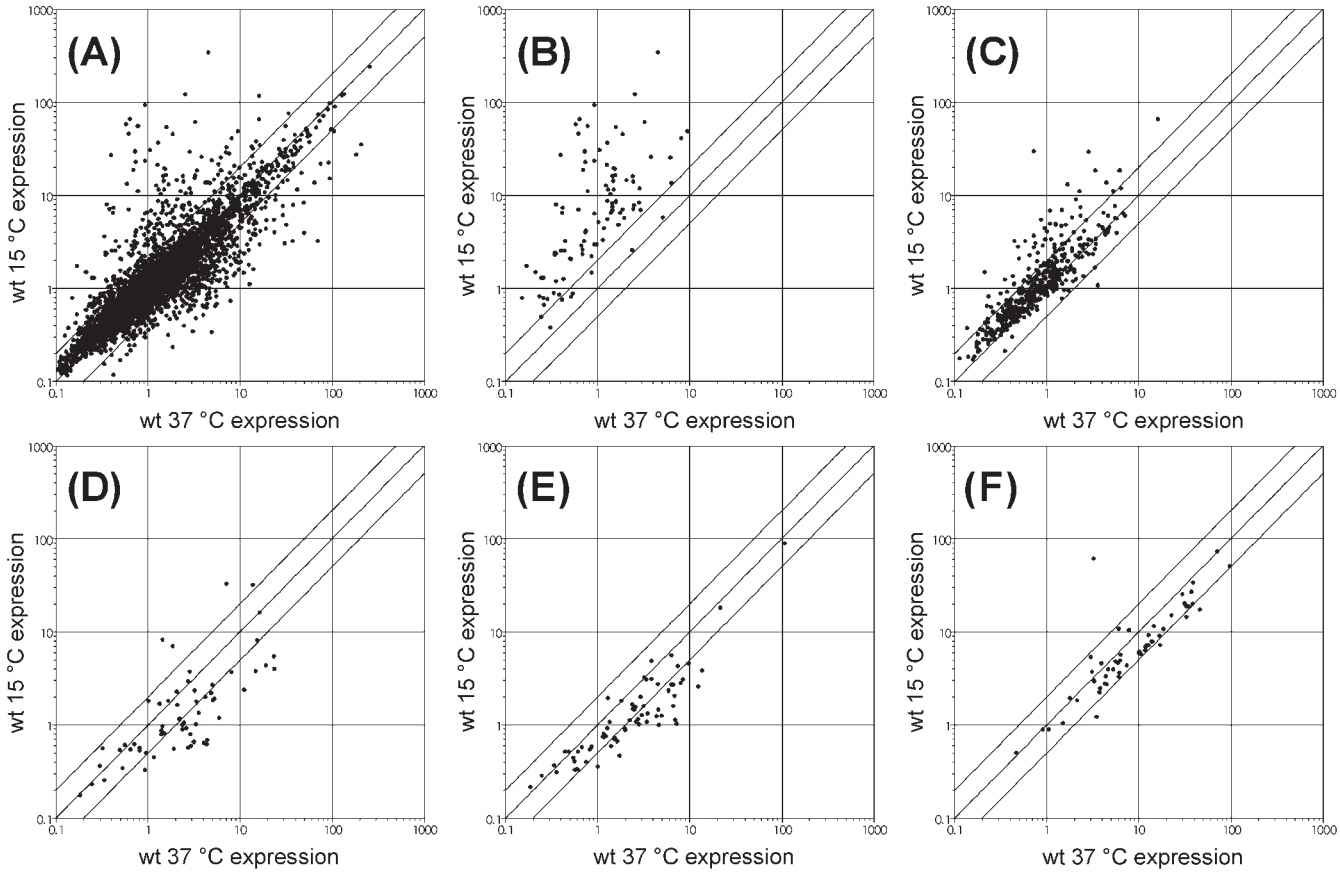


**Fig. 1.** Influence of chill stress on the transcriptional pattern of the *B. subtilis* wild-type (wt) strain 168. The scatter diagrams display normalized spot intensities of array hybridizations with RNA isolated from cells grown at 37 °C (horizontal axis) or 15 °C (vertical axis). For the presentation, spot intensities of the 4107 genes covered by the Panorama *B. subtilis* DNA macroarrays from Sigma Genosys Ltd have been normalized, and the duplicate spots of the four filters hybridized for each condition have been averaged as described in Methods. The two lines flanking the diagonal indicate a twofold change in expression. The following groups of genes are emphasized: (A) all 4107 protein-encoding genes of *B. subtilis* that are contained on the DNA array used; (B) the 100 SigB-dependent general stress genes whose stress induction has been described as exclusively dependent on SigB activity by Petersohn *et al.* (2001); (C) the 439 members of the sporulation-specific SigF, SigE, SigG and SigK regulons described by Steil *et al.* (2005); (D) the members of the SigW regulon, as described by Huang *et al.* (1998) and Wiegert *et al.* (2001); (E) genes implicated in chemotaxis and motility (Aizawa *et al.*, 2002); (F) genes encoding components of the ribosome (http://www.systems-biology.org/001/kegg/bsu.html).

**Table 1.** Low-temperature induced genes

| Gene* | Ratio† | Function‡ | Known regulators§ |
|---|---|---|---|
| *yabE* | 4·2 | Similar to cell wall-binding protein | |
| *ksgA* | 3·8 | Dimethyladenosine transferase | |
| *ctc* | 19·5 | General stress protein | SigB |
| *spoVC* | 3·8 | Probable peptidyl-tRNA hydrolase | SigB |
| *yabK* | 2·1 | Unknown | SigF |
| *mfd* | 2·1 | Transcription-repair coupling factor | |
| *divIC* | 2·0 | Cell-division initiation protein (septum formation) | SigE |
| *spoIIE* | 3·4 | Serine phosphatase ($\sigma^F$ activation)/asymmetric septum formation | Spo0A |
| *yabS* | 2·1 | Unknown | |
| *ctsR* | 2·4 | Transcriptional repressor of class III stress genes | SigB |
| *mcsA* | 2·4 | Modulator of CtsR repression | SigB |
| *mcsB* | 3·0 | Modulator of CtsR repression | SigB |
| *clpC* | 2·9 | Class III stress-response-related ATPase | SigB |
| *kbaA* | 2·1 | Activation of the KinB signalling pathway to sporulation | SigE |
| *ybcC* | 2·5 | Unknown | |
| *cypC* | 3·0 | Fatty acid beta-hydroxylating cytochrome P450 | SigB |
| ***ybyB*** | 49·0 | Unknown | SigB |
| *mpr* | 5·4 | Extracellular metalloprotease | SigE, DegU |
| *ycbJ* | 2·4 | Similar to macrolide 2′-phosphotransferase | |
| ***ycbK*** | 3·3 | Similar to efflux system | SigB |
| *ycbP* | 11·8 | Unknown | SigB, |
| *cwlJ* | 2·1 | Cell wall hydrolase (sporulation) | SigE, SigK |
| *lmrA* | 2·6 | Transcriptional repressor of the lincomycin operon | |
| ***ycdF*** | 10·9 | Similar to glucose 1-dehydrogenase | SigB |
| *ycdG* | 3·2 | Similar to oligo-1,6-glucosidase | SigB |
| *nadE* | 5·5 | $NH_3$-dependent $NAD^+$ synthetase | SigB |
| *ycgM* | 2·2 | Similar to proline oxidase | Spo0A, CodY |
| *yckD* | 2·1 | Unknown | SigF, SigG |
| *gerKC* | 2·0 | Germination response to the combination of glucose, fructose, L-asparagine and KCl | SigG |
| ***ydaD*** | 106·6 | Similar to alcohol dehydrogenase | SigB |
| ***ydaE*** | 28·0 | Unknown | SigB |
| *ydaF* | 2·3 | Similar to acetyltransferase | SigB |
| ***ydaG*** | 13·4 | Similar to general stress protein | SigB |
| ***ydaP*** | 4·5 | Similar to pyruvate oxidase | SigB |
| ***ydaT*** | 4·3 | Unknown | SigB |
| *ydbA* | 3·8 | Similar to ABC transporter (binding protein) | |
| *gsiB* | 78·7 | General stress protein | SigB |
| *ydbB* | 5·5 | Unknown | |
| *ydbD* | 23·3 | Similar to manganese-containing catalase | SigB |
| *rsbV* | 6·9 | Positive regulator of $\sigma^B$ activity (anti-anti-sigma factor) | SigB |
| *rsbW* | 7·0 | Negative regulator of $\sigma^B$ activity (switch protein/serine kinase, anti-sigma factor) | SigB |
| *sigB* | 6·0 | RNA polymerase general stress sigma factor | SigB |
| *rsbX* | 4·8 | Indirect negative regulator of $\sigma^B$ activity (serine phosphatase) | SigB |
| *yddT* | 4·5 | Unknown | DegU |
| *ydeB* | 3·2 | Similar to transcriptional regulator | |
| *ydjO* | 4·8 | Unknown | SigW |
| *yebE_2* | 2·9 | Unknown | |
| *yerI* | 3·3 | Similar to proteins | |
| *cotJB* | 2·2 | Polypeptide composition of the spore coat | SigE |
| *cotJC* | 3·3 | Polypeptide composition of the spore coat | SigE |
| *yesJ* | 2·1 | Unknown | SigE |
| ***yfnI*** | 7·1 | Similar to anion-binding protein | SigB |
| *yfmH* | 4·7 | Unknown | |
| *yfmB* | 3·0 | Unknown | |

**Table 1.** cont.

| Gene* | Ratio† | Function‡ | Known regulators§ |
|---|---|---|---|
| *yfmA* | 2·5 | Unknown | SigB |
| *yflT* | 73·4 | Unknown | SigB |
| *yflJ* | 2·5 | Unknown | SigE |
| ***yflH*** | 4·7 | Unknown | SigB |
| ***yflA*** | 4·1 | Similar to amino acid carrier protein | SigB |
| *yfkT* | 2·9 | Similar to spore germination protein | SigB |
| *yfkM* | 17·3 | Unknown | SigB |
| *yfkJ* | 10·0 | Similar to protein-tyrosine phosphatase | SigB |
| *yfkI* | 14·7 | Unknown | SigB |
| *yfkH* | 2·6 | Unknown | SigB |
| *yfkF* | 2·8 | Similar to multidrug-efflux transporter | |
| *yfkD* | 4·7 | Unknown | SigB, SigG |
| *sspH* | 3·7 | Small acid-soluble spore protein (minor) | SigG |
| ***yfhD*** | 4·9 | Unknown | SigB, SigF, SigG |
| ***yfhF*** | 3·0 | Similar to cell-division inhibitor | SigB |
| ***yfhK*** | 29·8 | Similar to cell-division inhibitor | SigB |
| ***yfhL*** | 3·9 | Unknown | SigB, SigW |
| *yfhM* | 5·9 | Similar to epoxide hydrolase | SigB, SigW |
| *sspE* | 4·2 | Small acid-soluble spore protein (major gamma-type SASP) | SigF, SigG |
| *ygaE* | 2·6 | Unknown | SigB, SigE |
| *prkA* | 2·2 | Serine protein kinase | SigE, SigK |
| *yhbH* | 3·6 | Unknown | SigE, SigK |
| ***yhcM*** | 7·5 | Unknown | SigB, SigF, SigG |
| *yhcN* | 2·6 | Unknown | SigF, SigG, SigK |
| *glpK* | 2·6 | Glycerol kinase | SigE |
| *ygxB* | 4·5 | Unknown | SigB |
| *spoVR* | 3·1 | Involved in spore cortex synthesis | SigE, SigK, DegU |
| *yhdF* | 2·2 | Similar to glucose 1-dehydrogenase | SigB |
| *yhdK* | 2·0 | Unknown | |
| *yhdN* | 9·1 | Similar to aldo-keto reductase | SigB |
| *yhdS* | 2·1 | Unknown | |
| *nhaX* | 34·7 | Putative regulatory gene for *nhaC* | SigB |
| *sspB* | 3·1 | Small acid-soluble spore protein (major beta-type SASP) | SigG |
| *yhaX* | 4·2 | Unknown | SigE |
| *aprE* | 2·7 | Extracellular alkaline serine protease (subtilisin E) | DegU |
| *yhfP* | 3·0 | Unknown | |
| *yhxC* | 4·0 | Similar to alcohol dehydrogenase | SigF, SigE |
| *yhzC* | 2·1 | Unknown | |
| *yhxD* | 8·0 | Similar to alcohol dehydrogenase | SigB |
| *yhjB* | 2·0 | Similar to metabolite permease | |
| *yisR* | 2·4 | Similar to transcriptional regulator (AraC/XylS family) | |
| *degA* | 2·2 | Transcriptional activator involved in the degradation of glutamine phosphoribosylpyrophosphate amidotransferase | |
| *yitT* | 10·7 | Unknown | SigB |
| *ipi* | 2·9 | Intracellular proteinase inhibitor | |
| *argB* | 2·2 | *N*-Acetylglutamate 5-phosphotransferase | |
| *argD* | 2·0 | *N*-Acetylornithine aminotransferase | |
| *carA* | 2·0 | Carbamoyl-phosphate transferase-arginine (subunit A) | |
| *appD* | 2·5 | Oligopeptide ABC transporter (ATP-binding protein) | CodY |
| *appA* | 2·4 | Oligopeptide ABC transporter (oligopeptide-binding protein) | SigW, CodY |
| *oppC* | 3·3 | Oligopeptide ABC transporter (permease) (initiation of sporulation, competence development) | |
| *oppD* | 3·3 | Oligopeptide ABC transporter (ATP-binding protein) (initiation of sporulation, competence development) | |

**Table 1.** cont.

| Gene* | Ratio† | Function‡ | Known regulators§ |
|---|---|---|---|
| *oppF* | 3·8 | Oligopeptide ABC transporter (ATP-binding protein) (initiation of sporulation, competence development) | |
| *yjbC* | 3·8 | Unknown | SigB |
| *yjbX* | 10·6 | Unknown | SigE |
| *yjcE* | 11·3 | Unknown | SigB |
| *yjcH* | 2·0 | Unknown | |
| *yjcO* | 2·0 | Unknown | Spo0A |
| *yjgB* | 3·5 | Unknown | SigB |
| *yjgC* | 30·1 | Similar to formate dehydrogenase | SigB |
| *yjgD* | 41·6 | Unknown | SigB |
| *rapA* | 7·4 | Response regulator aspartate phosphatase | Spo0A, DegU, CodY |
| *phrA* | 4·9 | Phosphatase (RapA) inhibitor | Spo0A |
| *yjpA* | 2·1 | Unknown | |
| *ykgA* | 5·4 | Unknown | SigB |
| *ykzA* | 104·2 | Similar to organic hydroperoxide resistance protein | SigB |
| *guaD* | 5·6 | Guanine deaminase | SigB |
| *ykrW* | 2·1 | Similar to ribulose-bisphosphate carboxylase | |
| *splB* | 2·3 | Spore photoproduct lyase | SigG |
| *ykwD* | 2·3 | Unknown | |
| *ykuF* | 3·3 | Similar to glucose 1-dehydrogenase | |
| *ykuL* | 2·0 | Unknown | Spo0A |
| *ykuS* | 3·3 | Unknown | SigF |
| *ykuT* | 3·0 | Unknown | SigB |
| *yknT* | 6·0 | Similar to sporulation protein $\sigma^E$-controlled | SigE |
| *ykzI* | 7·2 | Unknown | SigB, SigK |
| *nprE* | 2·1 | Extracellular neutral metalloprotease | DegU |
| *ylbA* | 2·5 | Unknown | |
| *ylbC* | 3·1 | Unknown | SigF |
| *ylbO* | 8·0 | Unknown | SigE |
| *murG* | 2·3 | UDP-*N*-acetylglucosamine-*N*-acetylmuramyl-(pentapeptide)pyrophosphoryl-undecaprenol *N*-acetylglucosamine transferase | SigE |
| *sigE* | 2·3 | RNA polymerase sporulation mother-cell-specific (early) sigma factor | Spo0A |
| *sigG* | 5·8 | RNA polymerase sporulation forespore-specific (late) sigma factor | SigF, SigG |
| *yloQ* | 2·2 | Unknown | |
| *nusA* | 2·1 | Transcription termination | |
| *ylxP* | 5·4 | Unknown | SigB |
| *rbfA* | 2·6 | Ribosome-binding factor A | SigB |
| *pnpA* | 2·0 | Polynucleotide phosphorylase (PNPase) | |
| *tepA* | 4·1 | Translocation-enhancing protein required for efficient pre-protein translocation | SigG |
| *ymfC* | 2·6 | Similar to transcriptional regulator (GntR family) | |
| *ymfJ* | 5·2 | Unknown | SigF, SigG |
| *spoVS* | 4·2 | Required for dehydratation of the spore core and assembly of the coat | |
| *ymcA* | 2·1 | Unknown | |
| *cotE* | 3·1 | Spore coat protein (outer) | SigE, SigK |
| *spoVK* | 2·7 | Disruption leads to the production of immature spores | SigE, SigK |
| *sspP* | 2·1 | Probable small acid-soluble spore protein (minor) | SigG |
| *sspO* | 2·5 | Small acid-soluble spore protein (minor) | SigG |
| *sspN* | 4·0 | Small acid-soluble spore protein (minor) | SigF, SigG |
| *tlp* | 2·7 | Small acid-soluble spore protein (thioredoxin-like protein) | SigF, SigG |
| *yngJ* | 2·4 | Similar to butyryl-CoA dehydrogenase | SigE, SigK |
| *yoxC* | 5·8 | Unknown | SigB |
| *yoaA* | 11·1 | Similar to ribosomal-protein-alanine *N*-acetyltransferase | SigB |
| *yoaT* | 2·6 | Unknown | |
| ***yocB*** | 16·0 | Unknown | SigB |

**Table 1.** cont.

| Gene* | Ratio† | Function‡ | Known regulators§ |
|---|---|---|---|
| *yocH* | 5·0 | Similar to cell wall-binding protein | Spo0A |
| *yocK* | 18·0 | Similar to general stress protein | SigB, SigE |
| *yozN* | 6·5 | Unknown | SigE |
| *yocN* | 6·5 | Similar to permease | SigE |
| *yozD* | 2·3 | Unknown | SigE, SigK |
| *yodM* | 2·3 | Unknown | |
| *yodL* | 2·0 | Unknown | |
| *yotH* | 3·0 | Unknown | |
| *yonU* | 2·1 | Unknown | |
| *yonD* | 2·2 | Unknown | |
| *yomL* | 3·9 | Unknown | DegU |
| *msrA* | 2·2 | Peptidyl methionine sulfoxide reductase | |
| *ypzA* | 2·1 | Unknown | SigG |
| *ypvA* | 3·0 | Similar to ATP-dependent helicase | |
| *trpB* | 2·8 | Tryptophan synthase (beta subunit) | |
| *trpF* | 2·5 | Phosphoribosyl anthranilate isomerase | |
| *trpD* | 2·2 | Anthranilate phosphoribosyltransferase | |
| *spoIVA* | 5·0 | Required for proper spore cortex formation and coat assembly | SigE |
| *ypeB* | 2·1 | Unknown | Sig |
| *sleB* | 2·5 | Spore cortex lytic enzyme | SigG |
| *ypbG* | 2·2 | Unknown | SigE, SigK |
| *sigF* | 2·5 | RNA polymerase sporulation forespore-specific (early) sigma factor | Spo0A |
| *spoIIAB* | 2·5 | Anti-sigma factor/serine kinase | Spo0A |
| *dacF* | 4·7 | Penicillin-binding protein (putative D-alanyl-D-alanine carboxypeptidase) | SigF, SigG |
| ***yqjL*** | 4·3 | Unknown | SigB |
| *bmrU* | 6·0 | Multidrug resistance protein cotranscribed with *bmr* | SigB |
| *bmr* | 7·0 | Multidrug-efflux transporter | SigB |
| *bmrR* | 7·3 | Transcriptional activator of the *bmrUR* operon | SigB |
| *spoIIIAG* | 2·0 | Mutants block sporulation after engulfment | SigE, SigK |
| *yqhP* | 2·5 | Unknown | SigB |
| *yqzG* | 2·2 | Unknown | SigF |
| *yqxL* | 7·0 | Unknown | SigB |
| *yqhA* | 2·8 | Similar to positive regulator of $\sigma^B$ activity | SigB |
| ***yqgZ*** | 32·0 | Unknown | SigB |
| *yqfX* | 4·1 | Unknown | SigG |
| *yrkH* | 2·7 | Unknown | |
| *yrrR* | 2·1 | Similar to penicillin-binding protein | SigF, SigE |
| *glnQ* | 2·2 | Glutamine ABC transporter (ATP-binding protein) | SigE |
| *glnH* | 3·3 | Glutamine ABC transporter (glutamine-binding protein) | SigE |
| *bofC* | 4·3 | Forespore regulator of the $\sigma^K$ checkpoint | SigB, SigF, SigG |
| *csbX* | 4·0 | $\sigma^B$-transcribed gene | SigB |
| *yrzG* | 2·3 | Unknown | |
| *coxA* | 3·2 | Spore cortex protein | SigG, SigE |
| *spoIIB* | 4·5 | Spatial and temporal regulation of the dissolution of septal peptidoglycan during engulfment | |
| *spoVID* | 3·0 | Required for assembly of the spore coat | SigE |
| *lonB* | 2·0 | Lon-like ATP-dependent protease | SigF |
| *ysnE* | 2·0 | Similar to acetyltransferase | SigE, SigK |
| *ysnF* | 70·6 | Unknown | SigB |
| *ysnB* | 2·6 | Unknown | SigE, SigG |
| *rph* | 2·1 | Ribonuclease PH | SigB, SigE |
| *uvrC* | 4·1 | Excinuclease ABC (subunit C) | SigK |
| *trxA* | 3·0 | Thioredoxin | SigB |
| *ysfC* | 4·8 | Similar to glycolate oxidase subunit | DegU |

**Table 1.** cont.

| Gene* | Ratio† | Function‡ | Known regulators§ |
|---|---|---|---|
| *argG* | 2·5 | Argininosuccinate synthase | |
| *argH* | 2·2 | Argininosuccinate lyase | |
| *ytfJ* | 4·5 | Unknown | SigF |
| *ytdI* | 4·1 | Unknown | |
| *sspA* | 5·6 | Small acid-soluble spore protein (major alpha-type SASP) | SigG |
| *yttP* | 2·1 | Unknown | Spo0A |
| *ytxG* | 5·3 | Similar to general stress protein | SigB |
| *ytxH* | 5·3 | Similar to general stress protein | SigB |
| *ytxJ* | 5·2 | Similar to general stress protein | SigB |
| ***dps*** | 7·8 | Stress- and starvation-induced gene controlled by $\sigma^B$ | SigB |
| *ytiA* | 10·8 | Similar to ribosomal protein | SigB, SigF |
| *ythC* | 3·4 | Unknown | |
| *menE* | 2·5 | *O*-Succinylbenzoic acid-CoA ligase | |
| *ytaB* | 3·5 | Unknown | SigB, SigK |
| *yuzA* | 3·6 | Unknown | SigB, SigG |
| *yuiD* | 2·0 | Unknown | SigB |
| *yumB* | 2·8 | Similar to NADH dehydrogenase | SigE |
| *paiA* | 19·4 | Transcriptional repressor of sporulation, septation and degradative enzyme genes | |
| *paiB* | 23·1 | Transcriptional repressor of sporulation and degradative enzyme genes | |
| *yutK* | 3·0 | Similar to $Na^+$/nucleoside cotransporter | |
| *yusL* | 2·0 | Similar to 3-hydroxyacyl-CoA dehydrogenase | DegU |
| *yvrE* | 7·3 | Similar to senescence marker protein-30 | SigB |
| *sspJ* | 3·1 | Small acid-soluble spore protein (minor) | SigG |
| *yvgO* | 42·5 | Unknown | SigB, SigK |
| *yvaA* | 12·7 | Unknown | SigB |
| *rnr* | 4·2 | Ribonuclease R | SigB |
| *yvaK* | 3·1 | Similar to carboxylesterase | SigB |
| *opuBD* | 2·6 | Choline ABC transporter (membrane protein) | |
| *sigL* | 2·2 | RNA polymerase sigma factor | |
| *uvrB* | 2·6 | Excinuclease ABC (subunit B) | |
| *uvrA* | 3·3 | Excinuclease ABC (subunit A) | |
| *yvjB* | 2·9 | Similar to carboxy-terminal processing protease | SigF, SigE, SigG |
| *yvyD* | 2·3 | Similar to $\sigma^{54}$ modulating factor of Gram-negative bacteria | SigB, Spo0A |
| *gtaB* | 3·0 | UTP-glucose-1-phosphate uridylyltransferase | SigB |
| *ywsB* | 26·1 | Unknown | SigB |
| *spoIIID* | 3·6 | Transcriptional regulator of $\sigma^E$- and $\sigma^K$-dependent genes | SigE |
| *ureA* | 3·9 | Urease (gamma subunit) | CodY |
| *ureB* | 4·7 | Urease (beta subunit) | CodY |
| *ureC* | 2·0 | Urease (alpha subunit) | CodY |
| *ywmF* | 3·2 | Unknown | SigB |
| ***csbD*** | 102·9 | $\sigma^B$-controlled gene | SigB |
| *moaA* | 2·2 | Molybdopterin precursor biosynthesis | |
| *spoIID* | 2·0 | Required for complete dissolution of the asymmetric septum | SigE, Spo0A |
| ***ywlB*** | 2·6 | Unknown | SigF, SigE, DegU |
| *spoIIR* | 2·6 | Required for processing of pro-$\sigma^E$ | SigF |
| *ywjC* | 5·2 | Unknown | SigB |
| *ywiE* | 2·3 | Similar to cardiolipin synthetase | SigB |
| *ywjA* | 3·7 | Similar to ABC transporter (ATP-binding protein) | SigB |
| *ywjB* | 4·2 | Unknown | SigB |
| *ywdL* | 2·1 | Unknown | SigF, SigE |
| *ywcI* | 5·1 | Unknown | |
| *sacT* | 7·0 | Transcriptional antiterminator involved in positive regulation of *sacA* and *sacP* | |
| *ywzA* | 25·4 | Unknown | SigB |

**Table 1.** cont.

| Gene* | Ratio† | Function‡ | Known regulators§ |
|---|---|---|---|
| *sacY* | 3·0 | Transcriptional antiterminator involved in positive regulation of levansucrase and sucrase synthesis | |
| *gspA* | 77·4 | General stress protein | SigB |
| *ywaF* | 2·6 | Unknown | SigB |
| *ywaC* | 2·9 | Similar to GTP-pyrophosphokinase | |
| *yxzF* | 2·6 | Unknown | SigB, SigK |
| *yxlJ* | 2·8 | Similar to DNA-3-methyladenine glycosidase | SigB |
| *katX* | 7·0 | Major catalase in spores | SigB, SigF |
| *scoA* | 2·0 | Probable succinyl CoA : 3-oxoacid CoA-transferase (subunit A) | SigE |
| *katE* | 2·4 | Catalase 2 | SigB |
| *csbC* | 3·8 | Putative sugar transporter | SigB |
| *yxbG* | 3·0 | Similar to glucose 1-dehydrogenase | SigB |
| *yxnA* | 3·1 | Similar to glucose 1-dehydrogenase | SigB |
| *yxaB* | 5·6 | Unknown | SigB |
| *yycD* | 6·6 | Unknown | SigB |
| *yyaT* | 2·5 | Unknown | SigK |
| *yyaH* | 2·4 | Unknown | |

*Genes whose deletion led to a cold-sensitive phenotype (Höper *et al.*, 2005) are shown in bold type. Operon structure is shown by indentation.
†Induction ratios between averaged per-Chip normalized intensities at 15 and 37 °C are displayed.
‡Function as defined in SubtiList database (http://genolist.pasteur.fr/SubtiList/).
§Known or suggested regulators are listed as defined by previous studies. These previous studies have investigated the following regulators: CodY (Molle *et al.*, 2003b), DegU (Mäder *et al.*, 2002; Ogura *et al.*, 2001), SigB (Helmann *et al.*, 2001; Petersohn *et al.*, 2001; Price *et al.*, 2001), SigE (Eichenberger *et al.*, 2003, 2004; Fawcett *et al.*, 2000; Feucht *et al.*, 2003; Steil *et al.*, 2005), SigF (Fawcett *et al.*, 2000; Steil *et al.*, 2005), SigG (Steil *et al.*, 2005), SigK (Eichenberger *et al.*, 2004; Steil *et al.*, 2005), SigW (Huang *et al.*, 1998; Petersohn *et al.*, 2001; Wiegert *et al.*, 2001), Spo0A (Fawcett *et al.*, 2000; Molle *et al.*, 2003a).

## Induction of the SigB-dependent general stress regulon during growth at low temperature

A recent proteome study by Brigulla *et al.* (2003) of chill-adapted *B. subtilis* cells revealed the induction of the SigB-dependent general stress response at low temperature (15 °C), and also demonstrated the chill induction of the *sigB* operon, which encodes the master regulator (SigB) and several regulators of its own activity (RsbV, RsbW, RsbX) (Kalman *et al.*, 1990). We therefore mined our transcriptome data for chill induction of members of the SigB regulon that had previously been defined through three independent transcriptional profiling studies (Helmann *et al.*, 2001; Petersohn *et al.*, 2001; Price *et al.*, 2001). Each of these SigB profiling studies assign between 120 and 190 genes to the SigB regulon, raising the potential size of this regulon to almost 300 members. However, only a limited number (approx. 25 %) of potential SigB-dependent genes are found in all three studies (Helmann *et al.*, 2001; Petersohn *et al.*, 2001; Price *et al.*, 2001).

Comparing our list of chill-induced genes (Table 1) with the data published by Petersohn *et al.* (2001) on the make up of the SigB regulon, we found 97 potential SigB-controlled genes among the chill-induced genes of *B. subtilis*. When the data of Helmann *et al.* (2001) and Price *et al.* (2001) were further considered, we found 82 and 92 SigB-controlled genes, respectively, among the low-temperature-induced genes. Taking all data into account, and removing the redundancy between the three studies, we now can assign approximately 40 % (112 of 279 genes) of the *B. subtilis* chill-induced genes to the SigB-dependent general stress regulon. Thus, the proteome study of Brigulla *et al.* (2003) and the data presented here conclusively demonstrate that chill stress is a potent inducer of the SigB regulon, and that the SigB-dependent general stress response is an integral part of the adaptation of the *B. subtilis* cell to growth at low temperature. This view is supported by the findings that a *sigB* mutant cannot effectively grow at low temperature (Brigulla *et al.*, 2003), that loss of SigB reduces the stationary-phase viability of cold-adapted cells (Mendez *et al.*, 2004), and that the disruption of individual members of the SigB regulon often leads to a cold-sensitive phenotype (Höper *et al.*, 2005). Of the 96 mutants with defects in individual general stress genes tested by Höper *et al.* (2005), 35 displayed a low-temperature-sensitive phenotype, and of these 35 mutated genes, 22 were found to be chill-induced in this study (Table 1). Many of the 112 SigB-dependent genes that are induced by chill stress have no assigned physiological function (Table 1). Even if one considers only those genes with a putative function, one cannot readily deduce how the products of these genes might contribute to the adaptation of *B. subtilis* to low temperature. It is thus

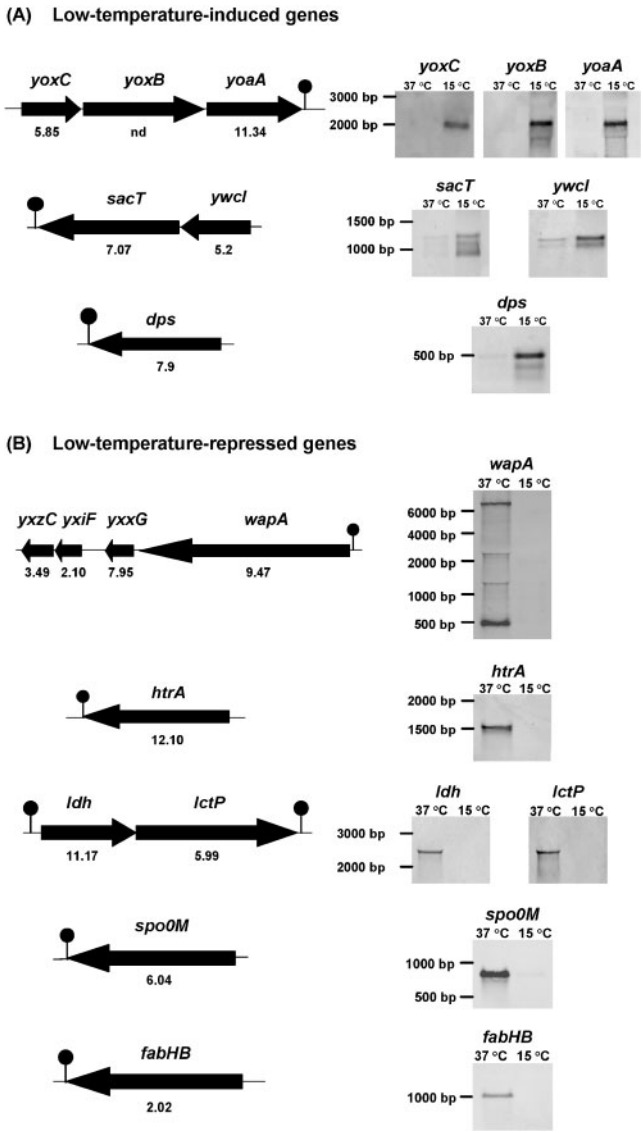


**Fig. 2.** Influence of low temperature on the expression pattern of *yoxCB*, *yoaA*, *sacT*, *ywcI*, *dps*, *wapA*, *htrA*, *ldh*, *lctP*, *spo0M* and *fabHB*. Total RNA was prepared as described in Methods from the *B. subtilis* wild-type strain 168 continuously grown in defined minimal medium at 37 or 15 °C. Equal amounts of RNA samples were electrophoretically resolved in agarose gels and transferred to nylon membranes. RNA blots were then hybridized with specific, digoxigenin-labelled probes internal to the structural genes of *yoxCB*, *yoaA*, *sacT*, *ywcI*, *dps*, *wapA*, *htrA*, *ldh*, *lctP*, *spo0M* and *fabHB*. Transcript sizes were estimated based on the relative positions of appropriate size markers. The schematic presentations on the left indicate the genetic organization/operon structure of the genes. Numbers below the arrows indicate the induction/repression ratios determined in the DNA array experiments; nd, the DNA array analysis did not yield statistically significant measurements for these genes. (A) Low-temperature-induced genes; (B) low-temperature-repressed genes.

obvious that detailed genetic, biochemical and physiological studies are required to reveal how these proteins are integrated into the low-temperature-adaptation network.

We find it worth commenting that we observed a different degree of matching between the chill-induced genes identified in this study (Table 1) and the assignment of the SigB regulon by the three previous transcriptional profiling studies (Helmann *et al.*, 2001; Petersohn *et al.*, 2001; Price *et al.*, 2001). Petersohn *et al.* (2001) discriminate between general stress genes whose stress induction is exclusively dependent on SigB activity and those genes that are also subjected to SigB-independent multiple stress induction. For the former group, 89 % (89 out of 100 genes) of the genes (Fig. 1B) were observed in this study to be induced by growth at low temperature, whereas for the latter group, only 33 % (8 out of 24 genes) were found to be so induced. Of the SigB-dependent genes proposed by Helmann *et al.* (2001) and Price *et al.* (2001), 65 and 48 %, respectively, were recovered if inspected for chill inducibility. This observation does not necessarily reflect quality differences in the different array studies, but rather appears to stem from the use of different DNA arrays. We and Petersohn *et al.* (2001) used the same type of commercially available DNA macroarray (Sigma Genosys Ltd), whereas Helmann *et al.* (2001) and Price *et al.* (2001) made use of different custom-synthesized DNA arrays.

## Chill stress triggers partial induction of the SigF, SigE and SigG sporulation regulons

Further inspection of the chill-induced *B. subtilis* genes (Table 1) led to the discovery that a substantial portion of these genes have previously been assigned by transcriptional profiling studies to regulons of the sporulation response (Eichenberger *et al.*, 2003, 2004; Fawcett *et al.*, 2000; Feucht *et al.*, 2003; Steil *et al.*, 2005). Contrary to that which has been observed for the SigB-dependent general stress regulon, for which most of the SigB-dependent genes are induced by chill stress (Fig. 1B), only a subgroup (15 %, 67 of 439 genes) of sporulation-specific genes (Steil *et al.*, 2005) is induced by chill stress (Fig. 1C). After initiation of sporulation (Hoch, 1995), the sporulation developmental programme progresses through the sequential induction of four regulons that are controlled by the alternative transcription factors SigF, SigE, SigG and SigK (Stragier & Losick, 1996). Based on the assignment of sporulation genes to the individual sporulation regulons, as described by Steil *et al.* (2005), we found 18 chill-induced genes among the 55 SigF regulon members, 28 among the 154 SigE regulon members, and 25 among the 113 SigG regulon members. None of the 132 SigK-controlled genes listed by Steil *et al.* (2005) was found among the low-temperature-induced genes identified by us. Comparison of the chill-induced genes defined in this study (Table 1) with other transcriptional profiling data that assess the sporulation response of *B. subtilis* (Eichenberger *et al.*, 2003, 2004; Fawcett *et al.*, 2000; Feucht *et al.*, 2003), assigned 14 additional chill-induced genes to the sporulation programme (Table 1). The

observation that only 81 of the 743 potential sporulation genes (Eichenberger *et al.*, 2003, 2004; Fawcett *et al.*, 2000; Feucht *et al.*, 2003; Steil *et al.*, 2005) were also chill induced (Table 1) indicates that the full sporulation programme is not implemented in cells growing at low temperature. This is consistent with the observation that spore formation is drastically reduced (approx. 1000-fold) in cells grown at 15 °C in comparison with cells growing at 37 °C (A. Bashir & U. Völker, unpublished results).

Partial induction of the sporulation regulons by chill stress raised the question of whether the enhanced transcription of these subgroups of the various *spo* regulons is dependent on the regular sporulation-specific transcription factors or is mediated by some unknown regulatory pathway. We chose three members (*cotE*, *spoIVA*, *yjbX*) of the chill-induced subgroup of the SigE regulon and performed Northern blot analysis on total RNA isolated from cells grown at 15 and 37 °C. As expected from the DNA array analysis (Table 1), transcription of each of these genes was induced by chill stress, and this induction was also observed in a *sigB* mutant (Fig. 3A). In contrast, chill induction of the *cotE*, *spoIVA* and *yjbX* genes was not detected in a mutant that lacks the first sporulation-specific sigma factor SigF (Fig. 3A). Taken together, these observations indicate that low-temperature induction of the small chill-induced fraction of the SigE regulon does indeed require a functional SigF protein.

The Northern blot analysis was also extended to selected members of the SigF (*yhcM*, *sigG*) and SigG (*sigG*, *sspE*) regulons. Low-temperature induction of these three genes was readily observable in the wild-type strain 168 and its isogenic *sigB* mutant (Fig. 3B). However, transcription still occurred in a *sigF* mutant when grown at low temperature (Fig. 3B). This persisting chill induction in the *sigF* mutant could perhaps be mediated by overlapping promoter recognition by SigB, SigG and SigF (Amaya *et al.*, 2001; Helmann & Moran, 2002). Chill induction of all three genes (*yhcM*, *sigG*, *sspE*) was still observable in a *sigB sigF* double mutant, but was lost upon the introduction of a defective *sigG* gene into the *sigB sigF* double-mutant strain (Fig. 3B). The dependence of low-temperature induction of the subgroups of the *spo* regulons on the sigma factors SigF and SigG provides evidence that regular signal transduction in the sporulation pathway is required to achieve this chill induction.

## Low-temperature induction of genes involved in the fine-tuning of Spo0A activity

Interestingly, the list of chill-stress-induced *B. subtilis* genes also contained genes encoding proteins involved in fine-tuning the phosphorylation status and activity of the master regulator (Spo0A) of the sporulation response and stationary-phase adaptation. The *rapA* and *phrA* genes displayed chill induction of 7·4- and 4·9-fold, respectively (Table 1). Northern blot analysis confirmed this induction and revealed cotranscription of the *rapA* and *phrA* genes under chill-stress conditions (Fig. 4B).

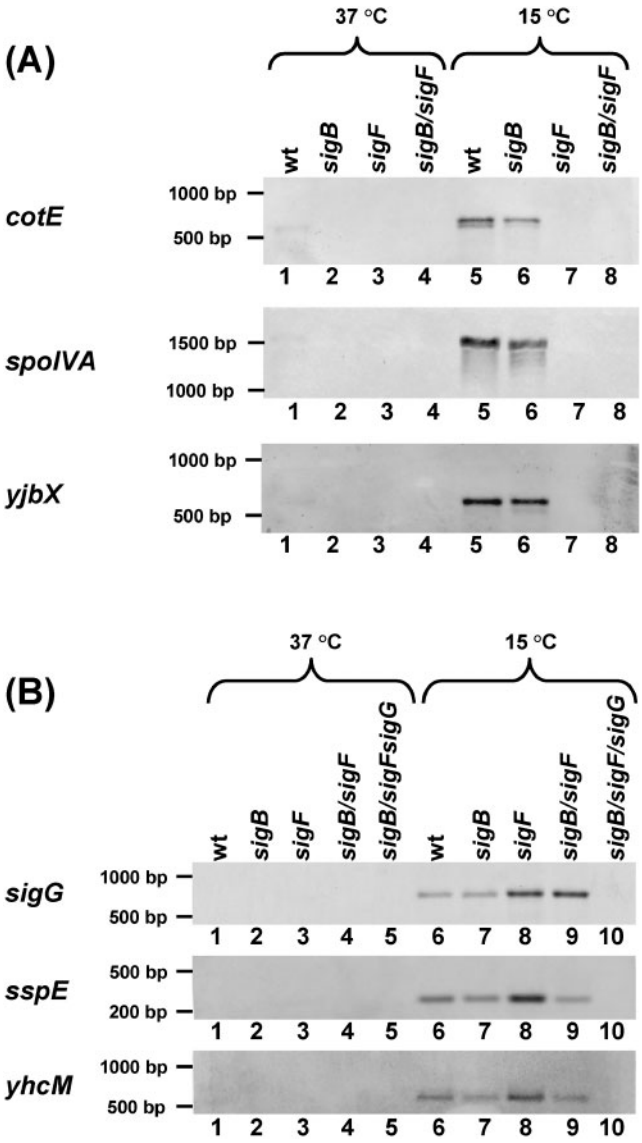


**Fig. 3.** Influence of low temperature on the expression pattern of selected members of the sporulation-specific SigE (A) and SigF/SigG (B) regulons. Total RNA was prepared from the *B. subtilis* wild-type (wt) strain 168 continuously grown in defined minimal medium at 37 or 15 °C. Equal amounts of RNA samples were electrophoretically resolved in agarose gels and transferred to nylon membranes. RNA blots were then hybridized with specific, digoxigenin-labelled probes internal to the structural genes of *cotE*, *spoIVA*, *yjbX*, *sigG*, *sspE* and *yhcM*. Transcript sizes were estimated based on the relative positions of appropriate size markers.

RapA is one of eleven Rap proteins that have been identified in *B. subtilis* by sequencing of its genome (Kunst *et al.*, 1997). This group of sequence-related proteins regulate protein activity either by acting as protein phosphatases that target the phosphorylated forms of response regulators of two-component systems (Perego *et al.*, 1996; Perego, 1999) or by specifically binding to regulatory proteins (Bongiorni *et al.*,

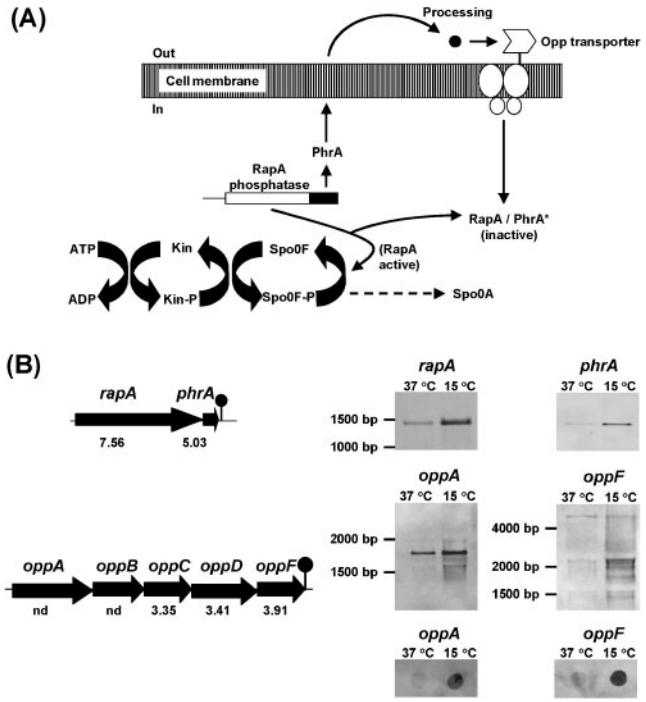


**Fig. 4.** Chill induction of genes encoding regulators of Spo0A activity. (A) Schematic presentation of the export–import control circuit for the PhrA pentapeptide that fine-tunes the phosphorylation status of the Spo0A response regulator by controlling the activity of the Spo0F∼P phosphatase RapA (adapted from Perego, 1999). (B) Northern blot analysis of the *rapA*, *phrA*, *oppA* and *oppF* genes. Total RNA was prepared from the *B. subtilis* wild-type strain 168 continuously grown in defined minimal medium at 37 or 15 °C. Northern blot analysis was performed as described in the legend to Fig. 2 with specific, digoxigenin-labelled probes internal to the structural genes of *rapA*, *phrA*, *oppA* and *oppF*. Transcript sizes were estimated based on the relative positions of appropriate size markers. For *oppA* and *oppF*, the Northern blot analysis was supplemented by a slot-blot analysis using the same probes. The schematic presentations on the left indicate the genetic organization/operon structure of the genes. Numbers below the arrows indicate the induction/repression ratios determined in the DNA array experiments; nd, the DNA array analysis did not yield statistically significant measurements for these genes.

2005; Core & Perego, 2003; Ogura *et al.*, 2003). The activity of seven of the eleven Rap proteins, including RapA, is regulated by specific pentapeptide inhibitors; these are generated through the export of the Phr protein from the cell, the proteolytic processing of this precursor protein and the subsequent reimport of a Phr-derived pentapeptide through the Opp oligopeptide transport system (see Fig. 4A) (Perego, 1997, 1999). The genes encoding the OppABC transport system (Perego *et al.*, 1991) were also induced by chill stress, as revealed by our transcriptional profiling data (Table 1) and the subsequent Northern blot and dot-blot analysis (Fig. 4B). Low-temperature induction was also observed for a second oligopeptide ATP-binding cassette (ABC)-type transport system, App (Table 1).

RapA, RapB and RapE specifically mediate the dephosphorylation of the Spo0F∼P intermediate of the sporulation phosphorelay that controls the phosphorylation status of the Spo0A response regulator (Jiang *et al.*, 2000; Perego *et al.*, 1994). In contrast to low-temperature induction of the *rapA* and *phrA* genes, the expression of the structural genes for the RapB and RapE proteins and the PhrE peptide did not change with decreasing temperature. None of the other eight *rap* genes was induced by chill stress; in fact, the genes for *rapF* and *rapH* were repressed.

The coordinated induction of the RapA/PhrA systems and the Opp oligopeptide transport system in chill-adapted cells indicates that the phosphorylation status of the Spo0A regulatory protein might need fine-tuning to ensure the survival of *B. subtilis* at low temperature. In this context, it is interesting to note that Mendez *et al.* (2004) have recently observed a dramatic (10 000-fold) decrease in stationary-phase survival of cold-adapted *B. subtilis* cells that are defective in Spo0A. These authors have also shown that the requirement for Spo0A for cellular survival at low temperature is independent of the transition-state regulator AbrB and does not rely on the ability of the cells to sporulate (Mendez *et al.*, 2004). In their study, Mendez *et al.* (2004) report induction of a transcriptional *spo0A–lacZ* fusion after a temperature shift from 37 to 20 °C. In contrast, our DNA array data did not show transcriptional induction of the *spo0A* gene in cells that were continuously cultured at 15 °C. We found by Northern blot analysis of total mRNA isolated from these chill-adapted cells that the *spo0A* mRNA was present at a level comparable to that in cells growing exponentially at 37 °C (Fig. 5A). However, when we assessed and quantified the level of the Spo0A protein in the *B. subtilis* wild-type strain 168 grown at 37 or 16 °C by a proteomic approach (see below), we found an accumulation of Spo0A in the low-temperature-adapted cells (Fig. 5B, C). This chill-induced accumulation of Spo0A was also apparent in an isogenic *sigB* mutant of *B. subtilis* strain 168 (Fig. 5C). Accumulation of the Spo0A protein has also been observed by Mendez *et al.* (2004) by Western blot analysis of wild-type cells shifted to 20 °C.

## Low-temperature induction of other physiological processes

The processes and regulons discussed thus far involve 193 out of the 279 chill-induced genes of *B. subtilis*. Of the remaining 86 genes, 33 have no ascribed function in the current SubtiList database (Moszer *et al.*, 2002). Eight genes (*sigE*, *sigF*, *spoIIAB*, *spoIIE*, *yocH*, *yttP*, *ycgM*, *ykuL*) (Table 1) have been shown to be subjected to positive control by the Spo0A regulator (Molle *et al.*, 2003a), some of which (*sigE*, *sigF*, *spoIIAB*, *spoIIE*) are involved in controlling the onset of sporulation and sporulation-specific gene expression (see above).

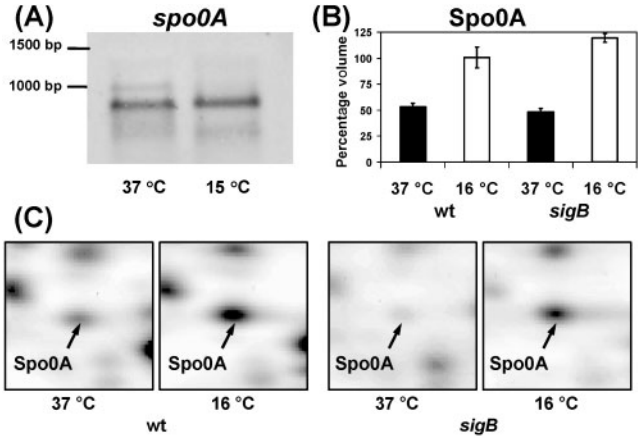


**Fig. 5.** Post-transcriptional regulation of Spo0A levels in low-temperature-grown *B. subtilis* cells. (A) Northern blot analysis of the *spo0A* gene. Total RNA was prepared from the *B. subtilis* wild-type (wt) strain 168 continuously grown in defined minimal medium at 37 or 15 °C. Northern blot analysis was performed as described in the legend to Fig. 2 with a specific, digoxigenin-labelled probe internal to the structural gene of *spo0A*. The transcript size was estimated based on the relative positions of appropriate size markers. (B) Quantitative evaluation of the Spo0A protein levels. Proteins extracts were prepared, labelled and separated by 2D-DIGE, as described in Methods. After matching of the corresponding gel images with the Delta2D software, Spo0A levels were quantified. The figure indicates the levels of Spo0A in cells of the *B. subtilis* wild-type strain 168 and its isogenic *sigB* mutant (BSM29) grown at 37 or 16 °C. (C) Regions of the two-dimensional gel images obtained from samples of the wild-type strain 168 and its isogenic *sigB* mutant (BSM29) grown at 37 or 16 °C. The spot corresponding to the Spo0A protein is indicated by the arrow.

The transcriptional profiling data revealed a 19-fold and a 23-fold chill induction of the *paiA* and *paiB* genes, respectively, which are cotranscribed as an operon, as assessed by Northern blot analysis (G. Streubel & E. Bremer, unpublished results). Overproduction of the PaiA and PaiB proteins from a multicopy plasmid has been shown elsewhere to reduce the formation of extracellular enzymes, including the alkaline serine protease AprE and the neutral metalloprotease NprE, and to trigger inhibition of sporulation at 37 °C (Honjo *et al.*, 1990). However, we found that the structural genes for the AprE and NprE proteases were moderately upregulated (Table 1) in chill-adapted *B. subtilis* cells, despite the strong induction of the *paiAB* operon, perhaps indicating that PaiAB exert their effects at the post-transcriptional level.

The list of chill-induced genes contained eight genes that code for additional regulatory proteins: YdeB, YisR, YmfC, DegA, LmrA, SacT, SacY and SigL. The alternative transcription factor SigL has previously been implicated in cold-shock adaptation of *B. subtilis* by Beckering *et al.* (2002), who identified the SigL-dependent transcriptional activator homologue YplP as a cold-shock-induced protein. These authors also demonstrate that a mutant strain with a gene disruption of *yplP* can only ineffectively recover from a cold shock. *B. subtilis* contains 36 histidine kinases and 34 response regulators of two-component regulatory systems (Fabret *et al.*, 1999). None of the genes encoding these two-component regulatory systems was induced by chill stress; in fact, the *resD*/*resE* and *degS*/*degU* genes were repressed.

In contrast to the general reduction in metabolism of chill-adapted cells that is described below, we noted specific induction of a few biosynthetic operons. We observed chill induction of five genes (*argB*, *argD*, *argG*, *argH*, *carA*) (Table 1) of the arginine biosynthetic pathway; the remaining four genes (*argC*, *argF*, *argJ*, *carB*) of this biosynthetic route were also slightly chill-induced, but did not pass the cut-off criteria adopted in this study. Interestingly, one of the operons (*ureA*, *ureB*, *ureC*) of the urea cycle that uses arginine as a substrate for ammonia generation was chill induced as well (Table 1). Furthermore, we observed induction of part of the tryptophan biosynthetic pathway: *trpB*, *trpD* and *trpF* were clearly induced by chill stress (Table 1), whereas *trpA*, *trpC* and *trpE* exhibited a moderate induction by chill stress, but did not fulfil our selection criteria.

Chill induction of the excision repair genes *uvrA*, *uvrB* and *uvrC* was also observed (Table 1). These three genes encode an excision nuclease complex that is involved in the repair of ultraviolet-light-induced pyrimidine dimers in DNA, indicating that there might be a greater need for DNA excision repair in cells grown at low temperature.

## Low-temperature-mediated repression of gene expression

Our transcriptional profiling data revealed that the transcription of 301 genes was repressed in *B. subtilis* cells grown at low temperature. Considering the potential functions of these genes, the transcriptional data indicate a general reduction in the metabolic activity of *B. subtilis* cells cultivated at low growth temperature. This is consistent with the strongly slowed growth of chill-adapted *B. subtilis* cells (Brigulla *et al.*, 2003). Among the processes that we found reduced in low-temperature-grown cells (15 °C) was the synthesis of components of the ribosome (Fig. 1F). The catabolic processes glycolysis, oxidative phosphorylation and ATP synthesis were all strongly reduced (Fig. 6), a finding which is compatible with a reduced energy demand of the cell during slow growth at low temperature. Anabolic activity was also affected, as exemplified by the overall reduction of the transcription of genes encoding enzymes involved in purine, pyrimidine, haem and fatty acid synthesis (Fig. 7). Of particular interest in this context is the reduced level of fatty acid synthesis, indicating that the cell has fully adjusted the lipid composition of the cytoplasmic membrane to sustain growth at the low temperature. This is consistent with our finding that the *des* gene encoding the Δ5-lipid desaturase was not induced during low-temperature

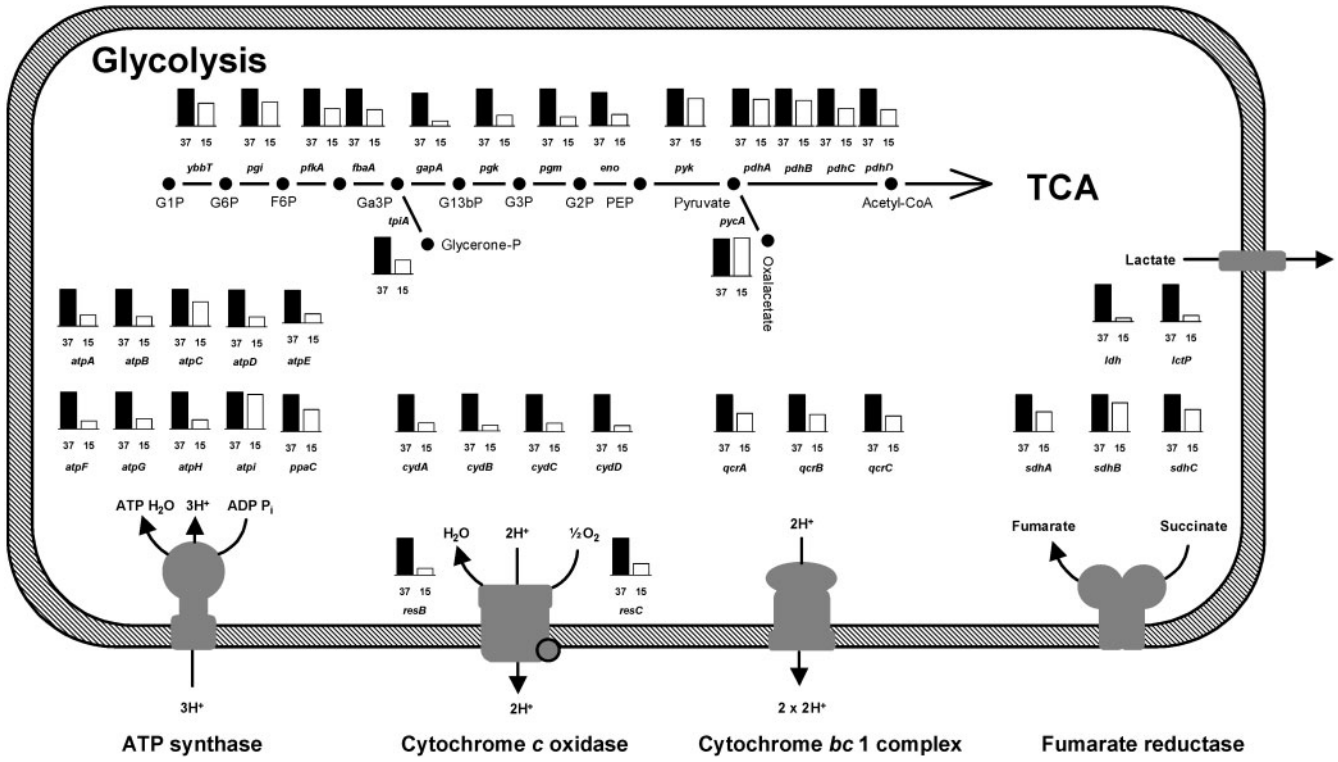


**Fig. 6.** Repression of catabolic functions of *B. subtilis* by chill stress. Selected components of catabolism, such as glycolysis, oxidative phosphorylation and ATP synthesis, as well as the expression pattern of the genes involved, are schematically presented. Expression levels that were observed by transcriptional profiling in cells of the *B. subtilis* wild-type strain 168 grown at 37 and 15 °C are represented by black and white bars, respectively. The expression values observed for the individual genes at 37 °C were set to 100 %.

growth. On the contrary, it was strongly upregulated following a cold shock (Aguilar *et al.*, 1998; Beckering *et al.*, 2002; Kaan *et al.*, 2002). The list of chill-stress-repressed genes contains approximately 50 additional genes with diverse metabolic functions, and another group of 59 genes of thus far undefined function.

Of the 71 genes implicated in chemotaxis and motility of *B. subtilis* (Aizawa *et al.*, 2002), the expression of 32 genes was reduced in the cold to a statistically significant extent (Fig. 1E). The reduction of the expression of chemotaxis and motility genes in general (Fig. 1E) suggests that the ability of cells to actively swim is reduced or abolished when *B. subtilis* is cultivated at low temperature. DegU ~ P is a major repressor of the motility genes in *B. subtilis* (Amati *et al.*, 2004). By matching our expression data with the assignments of the DegS/DegU-controlled regulon of Ogura *et al.* (2001) and Mäder *et al.* (2002), we were able to ascribe 28 of the 32 chill-repressed genes with a predicted function in chemotaxis and motility to the DegS/DegU regulon. The influence of the DegS/DegU two-component system on chill-repressed genes is not confined to the group of chemotaxis and motility genes, but extends to 35 additional cold-repressed genes.

The SigW regulon of *B. subtilis* has been functionally implicated in cell-wall-associated processes and the adaptation to alkaline shock (Cao *et al.*, 2002; Huang *et al.*, 1998; Wiegert *et al.*, 2001). Based on the definition of the SigW regulon structure of Huang *et al.* (1998) and Wiegert *et al.* (2001), we found 26 of the 62 SigW regulon members to be repressed at low temperature (Fig. 1D). Interestingly, among these were the structural genes for the central regulator of this regulon (*sigW*) and its repressing anti-sigma factor (*rsiW*) (Schobel *et al.*, 2004). Furthermore, a partial repression of the *comK* regulon (31 of 164 genes) (Berka *et al.*, 2002), controlling the development of competence for DNA uptake by *B. subtilis*, was observed in cells grown at low temperature. Among the chill-stress-repressed genes were four additional genes (*cggR*, *hbs*, *yvbA*, *yrxA*) that code for proteins involved in the regulation of gene expression.

A temperature-dependent gene expression was observed for the *ytrABCDEF* and *bkdR-ptb-pcd-buk-lpdV-bkdAA-bkdAB-bkdB* gene clusters. While both gene clusters have previously been shown to be induced following a cold shock (Beckering *et al.*, 2002; Nickel *et al.*, 2004), our transcriptome analysis of fully chill-stress-adapted *B. subtilis* cells revealed their repression. The *ytrABCDEF* gene cluster

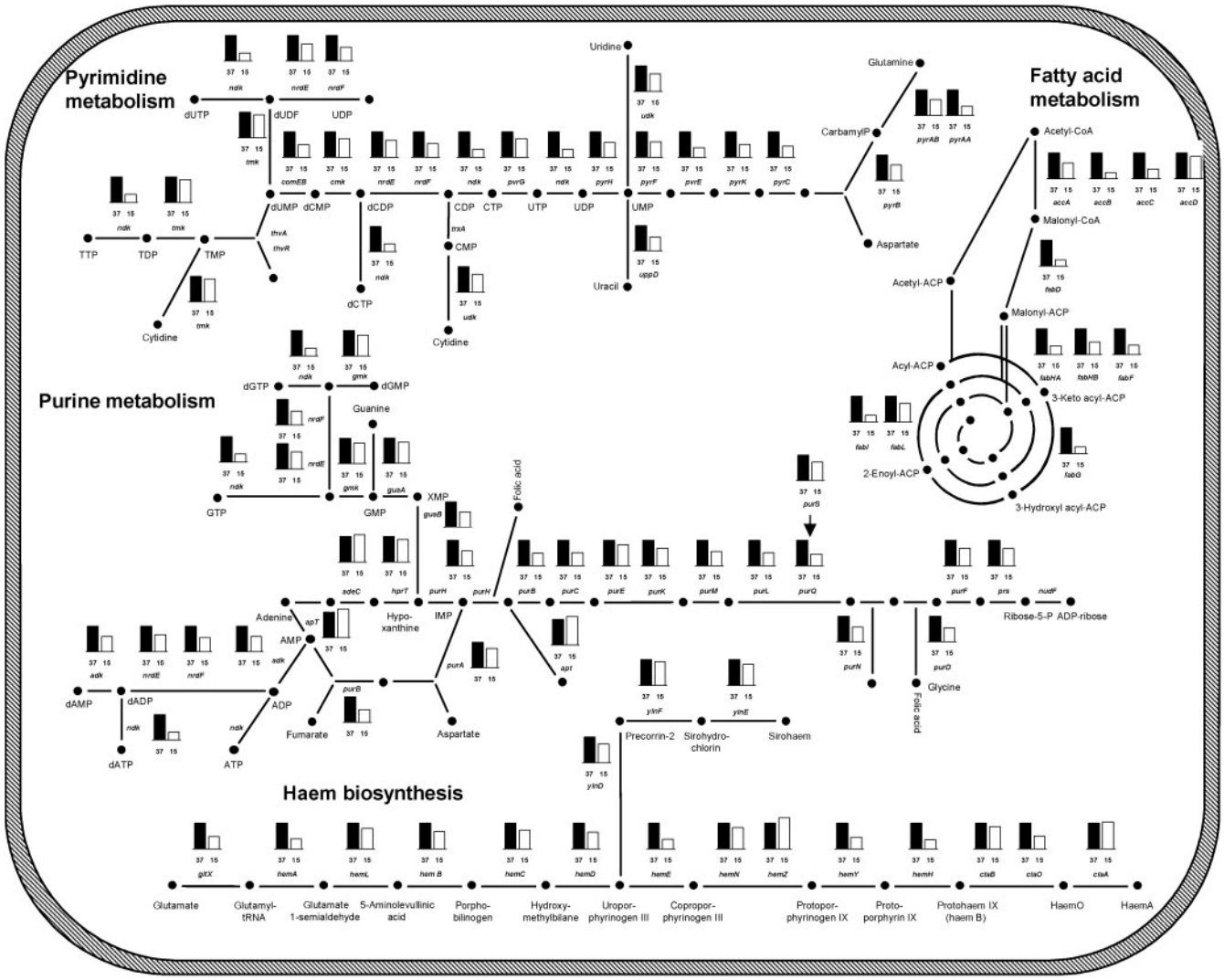


**Fig. 7.** Repression of anabolic functions of *B. subtilis* by chill stress. Selected components of anabolism, such as purine, pyrimidine, haem and fatty acid biosynthesis, as well as the expression pattern of the genes involved, are schematically presented. Expression levels that were observed by transcriptional profiling in cells of the *B. subtilis* wild-type strain 168 grown at 37 and 15 °C are represented by black and white bars, respectively. The expression values observed for the individual genes at 37 °C were set to 100 %.

encodes a repressor (*ytrA*) and the components of an ABC transporter for acetoin utilization (Yoshida *et al.*, 2000). The *bkdR-ptb-pcd-buk-lpdV-bkdAA-bkdAB-bkdB* gene cluster encodes enzymes that catalyse the degradation of branched-chain amino acids, which are necessary for the maintenance of membrane fluidity at low temperatures; its transient induction subsequent to cold shock is primarily due to stabilization of its mRNA following temperature downshift (Nickel *et al.*, 2004).

The *opuAA-opuAB-opuAC* operon that encodes the components of an ABC transport system (OpuA) for the compatible solute glycine betaine (Kempf & Bremer, 1995) is repressed at low temperature. However, the OpuA transporter makes an important contribution to the overall uptake of glycine betaine (Bremer, 2002) when *B. subtilis* cells accumulate this compatible solute as a cryoprotectant (T. Hoffmann & E. Bremer, unpublished results). Thus, in chill-adapted cells, appropriate functioning of the OpuA ABC transport system might be ensured by post-transcriptional regulation or by control of its activity.

## Proteome analysis of chill-adapted *B. subtilis* cells

Low-temperature incubation of micro-organisms imposes severe constraints on translation due to the increased formation of mRNA secondary structures. One of the protective adaptation responses of the *B. subtilis* cell to cold shock specifically circumvents this problem by synthesizing

cold-shock proteins that act as RNA chaperones (Graumann & Marahiel, 1996; Weber & Marahiel, 2002) or by inducing the synthesis of RNA helicases that are involved in the unwinding of double-stranded RNA regions (Beckering *et al.*, 2002; Jones *et al.*, 1996). Thus, post-transcriptional regulatory processes are particularly important in adjusting translation and thereby ensuring appropriate protein levels for the low-temperature adaptation of microbial cells.

We therefore supplemented our transcriptional profiling approach for low-temperature-grown *B. subtilis* cells with a proteomic study. For this purpose, we grew the *B. subtilis* wild-type strain 168 at either 37 or 16 °C to mid-exponential phase ($OD_{578}$ of 1) and prepared from these cells total soluble protein extracts for analysis by 2D-DIGE. Matching of the protein patterns by the Delta2D software allowed the assignment of 1085 protein spots. Of these protein spots, 491 exhibited statistically significant differences (twofold) between cells grown at 37 and 16 °C: 246 protein spots were chill-induced and 245 were chill-repressed. The dual-channel image overlay of the protein profiles that is presented in Fig. 8 gives an impression of the massive temperature-dependent changes in the cytosolic protein profile of *B. subtilis*. We identified 109 induced and 88 repressed protein spots by mass spectrometry that were subsequently allocated to 86 and 60 distinct protein species, respectively. Detailed information on the identification of proteins by mass spectrometry and the quantification of the protein levels is given in Supplementary Tables 3 and 4.

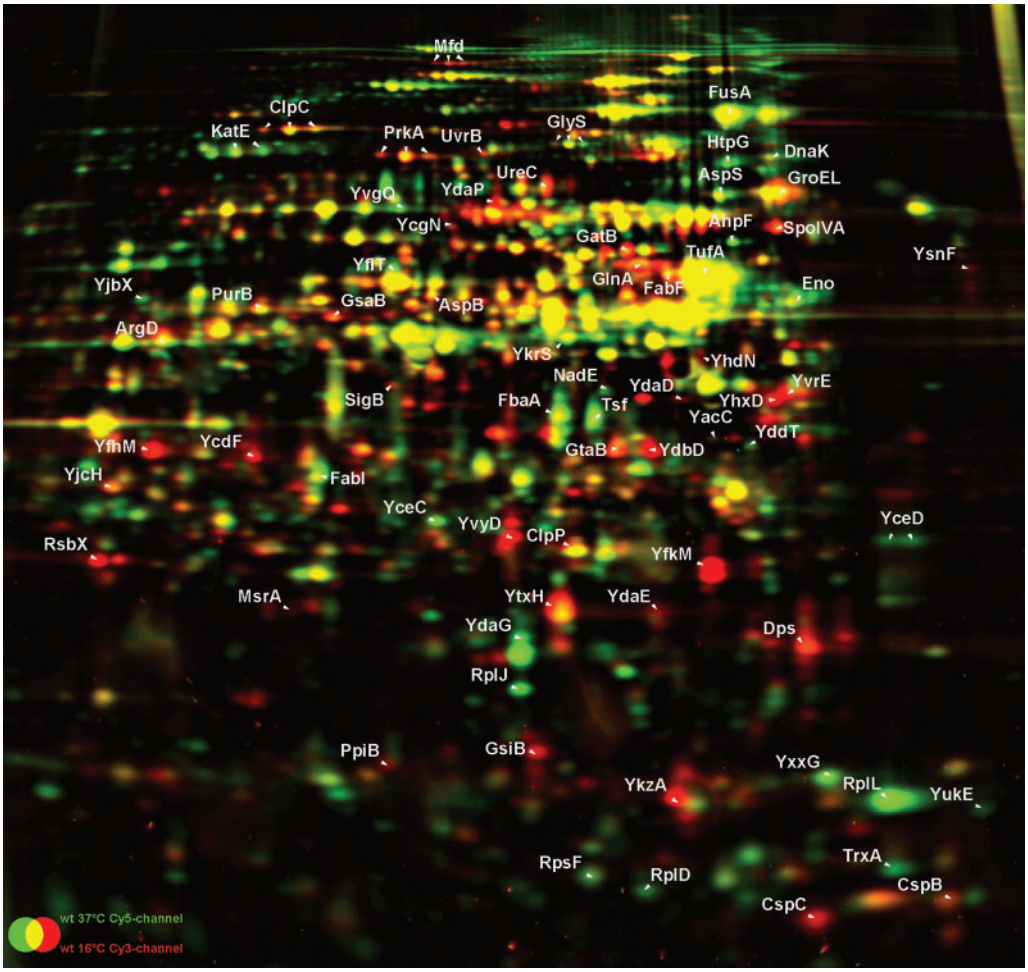


**Fig. 8.** 2D-DIGE analysis of chill adaptation of the *B. subtilis* wild-type strain 168. Crude protein extracts were prepared, labelled and separated by 2D-DIGE, as described in Methods. After scanning the intensities of the fluorescence staining of the Cy2, Cy3 and Cy5 dyes with a Typhoon scanner, images were analysed and quantified with the Delta2D software package. A representative dual-channel overlay image of the protein profile of cells of the *B. subtilis* wild-type strain 168 grown at either 37 or 16 °C is displayed. Protein spots present at higher levels at 37 °C are displayed in shades of green, those present at increased levels at 16 °C appear in red, and those whose level does not change in response to temperature are shown in yellow. Proteins were identified by MALDI-MS-MS mass spectrometry. Protein spots induced or repressed by low-temperature incubation are labelled.

A comparison of the groups of chill-induced genes (Table 1) and the proteins found at increased levels in low-temperature-grown cells (Supplementary Table 3) revealed an overlap of 28 entries. Consistent with the strong induction of the SigB regulon (Fig. 1B) and the preponderance of its members among the chill-induced genes (Table 1), 20 of the 28 entries were allocated to the SigB-dependent general stress response (Fig. 8). As expected, these members of the SigB regulon were not found as chill-induced proteins when a proteome analysis was carried out with a *sigB* mutant derivative of the *B. subtilis* wild-type strain 168. Examples of the comparison of the wild-type strain 168 and its isogenic *sigB* mutant are shown in Fig. 9(A). The remaining eight proteins that were chill induced and whose structural genes displayed transcriptional induction at low temperature were UvrB, SpoIVA, MsrA, UreC, Mfd, YjcH, ArgD and PrkA (Fig. 8).

We found 58 proteins that were chill induced (Supplementary Table 3) but whose structural genes did not show a corresponding increase in transcription. Notably, this group of proteins contained the cold-shock proteins CspB and CspC, which were chill induced 2·5-fold and 16-fold, respectively (Fig. 9B). It is well established that the production of cold-shock proteins occurs mainly at the post-transcriptional level, whereas transcriptional activation of the corresponding structural genes (*csp*) is only modest (Beckering *et al.*, 2002; Graumann *et al.*, 1996; Kaan *et al.*, 1999). Also among the chill-induced proteins was the peptidyl-prolyl isomerase PpiB, which contributes to proper protein folding. PpiB has previously been identified in a proteome study as a cold-shock protein (Graumann *et al.*, 1996). The authors of the Graumann *et al.* (1996) study also showed that the heat-shock proteins GroES and ClpP are synthesized at reduced levels immediately following temperature downshift. In our proteomic appraisal of cells grown under chill stress, we now observed increased levels of both GroEL and ClpP (Fig. 8). This is consistent with a recent analysis of the long-term cold adaptation of *Listeria monocytogenes*, which displays increased *clpP* and *groEL* mRNA levels in the cold (Liu *et al.*, 2002). Interestingly, we also noted increased levels of enzymes (GatB, GlnA, GsaB, YcgN) involved in glutamine/glutamate metabolism among the low-temperature-induced proteins. Since we also observed an upregulation of genes encoding components (*glnH*, *glnQ*) of an ABC transport system for glutamine (Table 1), increased levels of glutamine might help the *B. subtilis* cell to cope with chill stress.

Fourteen (AhpF, Eno, FbaA, FabI, RpsF, RplJ, RplL, Tsf, YukE, YvgQ, YcgR, YxxG, YceC, YceD) of the 60 proteins that were present at decreased levels at 16 °C also displayed reduced expression of their coding genes during low-temperature growth. When the proteins that displayed decreased levels in cultures propagated at low temperature were assigned to functional categories, a reduction in the level of components of the translational apparatus (RpsF, RplJ, RplL, RplD, Tsf, FusA, TufA, AspS, GlyS, YkrS) and reduced levels of stress proteins (DnaK, HtpG, YacC) were observed. The remaining group of proteins displaying reduced levels in chill-stressed cultures was not large enough

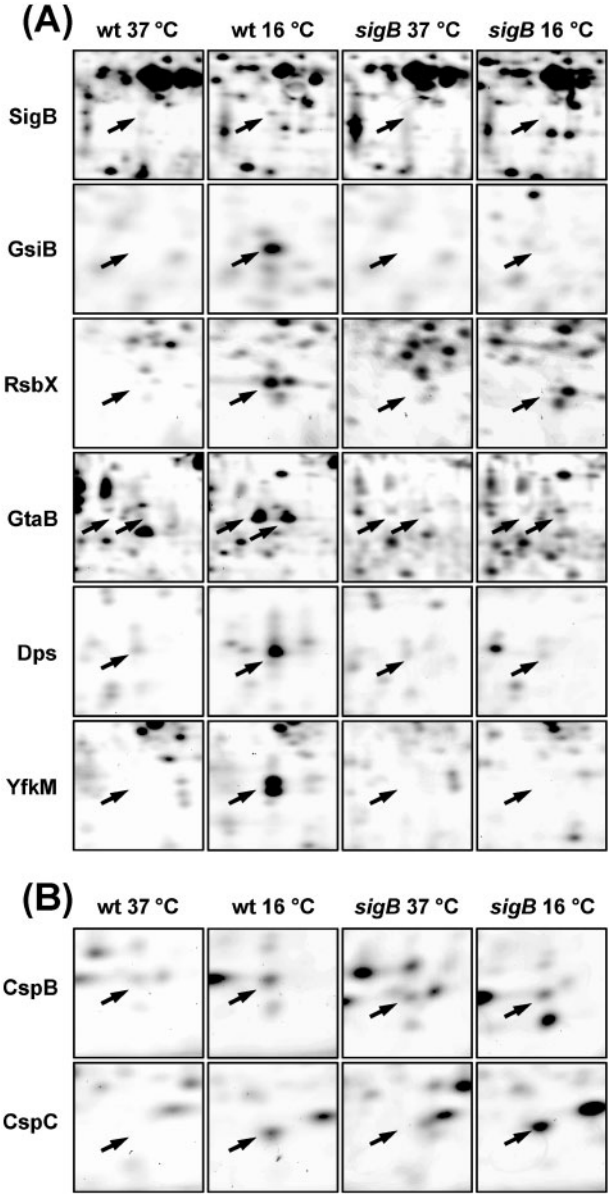


**Fig. 9.** Comparative analysis of the levels of general stress proteins and cold-shock proteins. Crude protein extracts were prepared, labelled and separated by 2D-DIGE, as described in Methods. After scanning the intensities of the fluorescence staining of the Cy2, Cy3 and Cy5 dyes with a Typhoon scanner, images were analysed and quantified with the Delta2D software package. Selected representative sections of the two-dimensional image of the protein profile of cells of the *B. subtilis* wild-type (wt) strain 168 and its isogenic *sigB* mutant (BSM29) grown at either 37 or 16 °C are displayed. (A) Expression levels of the general stress proteins SigB, GsiB, RsbX, GtaB, Dps and YfkM; (B) expression levels of the cold-shock proteins CpsB and CspC. Arrows point towards the spots for the respective proteins.

to significantly cover entire biochemical pathways, but the enzymes included in this list support the notion of our transcriptional profiling study, which indicates a reduced need for metabolic activity, including purine and pyrimidine biosynthesis (Fig. 7), in *B. subtilis* cells grown at low temperature.

In a few cases, we observed a discrepancy between the transcriptional profiling data and the data obtained from the proteome analysis. This category contains the PurB, FabF, AspB and GlnA proteins that were upregulated in the proteome analysis and whose structural genes were downregulated in the transcriptional profiling experiment (Supplementary Tables 2 and 3). Conversely, the levels of the KatE, TrxA, YdaG, NadE, YjbX and YddT proteins were reduced in chill-adapted cells, but their mRNA levels exhibited chill induction in the DNA array analysis (Table 1 and Supplementary Table 4). These particular observations, as well as the substantial number of proteins displaying differential levels in the proteome analysis yet whose corresponding genes were not differentially expressed in the transcriptional profiling study (94 of a total of 149 differentially expressed proteins), reinforce the view that post-transcriptional regulatory events strongly influence the protein pattern of chill-adapted *B. subtilis* cells.

### Cold shock and low-temperature growth constitute two different phases of the chill adaptation of *B. subtilis*

Recently, two independent transcriptional profiling studies that assess the cold-shock adaptation of *B. subtilis* have been published (Beckering *et al.*, 2002; Kaan *et al.*, 2002). These gave us the opportunity to compare the initial adaptation response of *B. subtilis* to a sudden drop in temperature with the long-term adaptation of cells grown at low temperature. In a first step, we compared the lists of cold-induced and cold-repressed genes reported by Beckering *et al.* (2002) and Kaan *et al.* (2002) with each other. Out of the 46 (Kaan *et al.*, 2002) and 114 (Beckering *et al.*, 2002) genes identified as cold-shock-induced genes, only five were found in both studies. Of the genes repressed by cold shock, only 24 genes were recognized by both studies: Beckering *et al.* (2002) reported 253, whereas Kaan *et al.* (2002) described 52. This surprisingly small overlap in the identification of temperature-regulated genes between the two studies may have resulted from the use of different *B. subtilis* strains and media, the imposition of a different extent of temperature downshift [37 °C down to 18 °C (Kaan *et al.*, 2002) or 15 °C (Beckering *et al.*, 2002)], and the utilization of DNA arrays from different vendors. We consider the use of different media, different temperature downshifts and different DNA macroarrays as the most important factors that influenced the different outcome of the two transcriptional profiling studies. Beckering *et al.* (2002) used the same DNA macroarrays as those employed in our study; the same type of medium was used, and a temperature downshift from 37 to 15 °C was employed. We grew our *B. subtilis* cultures for the DNA array studies at 15 °C. Consequently, here, we confine to the study of Beckering *et al.* (2002) our comparison of the adaptive response of *B. subtilis* to growth at low temperature and the initial stress response of this micro-organism to a cold shock. In addition, we noticed little overlap between our study and that of Kaan *et al.* (2002): there were only two low-temperature-induced and seven low-temperature-repressed genes in common.

Cold shock transiently reduces the growth of *B. subtilis* (Weber & Marahiel, 2003), and cultivation at low temperature permits only slow growth (Brigulla *et al.*, 2003). Thus one would expect that this reduction in growth would be reflected in the gene expression profile of both phases of chill adaptation. Indeed, we observed an overlapping group of 85 cold-repressed genes in both phases (Supplementary Table 5), whereas 168 genes were repressed only by cold shock, and 216 genes were repressed only by cultivation at low temperature. The most obvious finding was a reduction in the expression of genes encoding enzymes required for ATP synthesis and oxidative phosphorylation. We also noted repression of the genes encoding the regulatory aspartate phosphatase RapF and its inhibitory peptide PhrF. Most of the remaining genes that are repressed by cold shock and low-temperature growth code for enzymes with diverse metabolic functions, indicative of a substantial reduction in metabolic activity.

Only 11 genes (*rbfA*, *ylxP*, *ywaC*, *paiAB*, *ydeB*, *ydjO*, *yjbC*, *yhdK*, *yhdS*, *trpF*) were induced both during the cold-shock response and in fully chill-adapted *B. subtilis* cells (for details see Supplementary Table 5). Among these genes was the structural gene (*rbfA*) that encodes the ribosome-binding factor A. The RbfA protein of *Escherichia coli* has been shown to bind to the 30S subunit of the ribosome, and this may affect translation initiation at low temperatures (Dammel & Noller, 1995). Furthermore, chill induction was observed for *paiA* and *paiB*; the products of these genes have been implicated in multicopy suppression of the production of extracellular enzymes and sporulation (Honjo *et al.*, 1990). We also noted the induction of the *ydeB* gene, whose gene product displays similarity to transcriptional regulators; nothing is known about the target genes for the YdeB protein. The rather large numbers of genes that were exclusively induced early (103 genes) or late (268 genes) in the adaptation process of *B. subtilis* to low temperature indicate the different physiological needs of the cell for these distinct phases of chill adaptation.

## Conclusions

Using a combined transcriptomic and proteomic approach, we have identified on a genome-wide scale the members of the chill-stress stimulon of *B. subtilis*. Both experimental approaches revealed massive temperature-dependent changes in the gene expression and the protein profile of chill-adapted *B. subtilis* cells. A total of 580 genes, representing approximately 14 % of the protein-coding capacity of *B. subtilis*, displayed temperature-dependent alterations: 279 genes were induced and 301 genes were repressed.

The transcriptional profiling approach employed in this study cannot distinguish between increased transcription initiation and mRNA stabilization by low temperature. Nevertheless, our data identified many new *B. subtilis* genes in chill-stress adaptation that were not previously known to be regulated by low temperature. This identification now provides the opportunity for targeted inactivation of genes regulated by chill stress and the analysis of corresponding mutant phenotypes to unravel the genetic and physiological processes that underlie the adaptation of *B. subtilis* to low temperature.

The comparative analysis of the transcriptional responses of cold-shocked *B. subtilis* cells (Beckering *et al.*, 2002) and those of cells grown at low temperature revealed that there are two distinctively different phases in the adaptation of *B. subtilis* to low temperature. Such a distinction has also been made for *B. subtilis* cells subjected to a salt shock or continuously growing at high salinity (Steil *et al.*, 2003). Important changes in low-temperature-grown *B. subtilis* cells include the almost complete induction of the SigB-controlled general stress regulon, partial induction of the sporulation-specific SigF, SigE and SigG regulons, and the induction of a regulatory circuit involved in the fine-tuning of the phosphorylation status and activity of the Spo0A response regulator. The bioinformatic analysis of genes that were repressed in chill-stress-grown cells revealed a general repression of genes involved in glycolysis, oxidative phosphorylation, ATP synthesis, purine and pyrimidine biosynthesis, and haem and fatty acid biosynthesis. Likewise, transcription of genes with predicted functions in chemotaxis and motility and members of the SigW regulon was strongly reduced. Taken together, these observations reflect the reduced catabolic and anabolic needs of slow-growing, low-temperature-cultivated *B. subtilis* cells.

The complementation of the transcriptional profiling by a proteomic approach allowed the identification of a substantial number of proteins induced or repressed in low-temperature-grown *B. subtilis* cells whose structural genes were not identified as differentially regulated by our transcriptional analysis. The rather large size of this group of proteins indicates that post-transcriptional regulatory phenomena make major contributions to the adaptation of *B. subtilis* to growth at low temperature.

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