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Chapter 2

Transcriptome Analysis of Sorbic Acid-Stressed *Bacillus subtilis* Reveals a Nutrient Limitation Response and Indicates Plasma Membrane Remodelling

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2.1. Abstract

The weak organic acid sorbic acid is a commonly used food preservative, as it inhibits the growth of bacteria, yeasts, and moulds. We have used genome-wide transcriptional profiling of *Bacillus subtilis* cells during mild sorbic acid stress to reveal the growth-inhibitory activity of this preservative and to identify potential resistance mechanisms. Our analysis demonstrated that sorbic acid-stressed cells induce responses normally seen upon nutrient limitation. This is indicated by the strong derepression of the CcpA, CodY, and Fur regulon and the induction of tricarboxylic acid cycle genes, SigL- and SigH-mediated genes, and the stringent response. Intriguingly, these conditions did not lead to the activation of sporulation, competence, or the general stress response. The fatty acid biosynthesis (*fab*) genes and BkdR-regulated genes are upregulated, which may indicate plasma membrane remodelling. This was further supported by the reduced sensitivity toward the *fab* inhibitor cerulenin upon sorbic acid stress. We are the first to present a comprehensive analysis of the transcriptional response of *B. subtilis* to sorbic acid stress.

2.2. Introduction

The food industry commonly utilizes sorbic acid and other weak organic acids as preservatives. Sorbic acid (*trans*, *trans*-2,4-hexadienoic acid) is a six-carbon unsaturated fatty acid with a pK_a of 4.76 and was first isolated from unripe berries of Rowan (*Sorbus aucuparia*). The acid, or its anionic salt, is used in a variety of food products and has a broad range of antimicrobial activities against spoilage bacteria, yeasts and moulds (Beales, 2004, Davidson, 2001, Piper *et al.*, 2001). However, the exact mechanism by which sorbate inhibits microbial growth is not entirely understood. No single mechanism appears to explain its toxicity to various spoilage organisms.

Depending on the pK_a of the acid and the pH of the environment, in solution sorbate exists in equilibrium between the dissociated (S^-) and undissociated (HS) state. The neutral HS is lipid permeable and able to diffuse into the cell, reaching an equilibrium when the inside and outside concentrations of HS are equal. Inside, a new equilibrium is formed between S^- and HS, releasing protons into the cytosol. This may acidify the cytosol, causing inhibition of many metabolic functions (Brown & Booth, 1991, Salmond *et al.*, 1984). Furthermore, the lipophilic tail of the sorbate molecule has been shown to disrupt the membrane and interfere with membrane proteins (Stratford & Anslow, 1998). This, together with the entry of protons could result in a loss of the proton motive force, disrupting oxidative phosphorylation and affecting transport of nutrients (Freese *et al.*, 1973, Ronning & Frank, 1987, Bauer *et al.*, 2003). Also, accumulation of S^- in the cell could cause a rise in osmolarity and affect cytosolic enzymes (Azukas *et al.*, 1961, York & Vaughn, 1964).

In order to counteract the effects of sorbic acid, microorganisms use various resistance mechanisms. *Saccharomyces cerevisiae* uses H^+ -ATPases to pump out the excess protons at the cost of ATP to maintain pH homeostasis (Holyoak *et al.*, 1996, Lambert & Stratford, 1999) and induces a dedicated ATP binding cassette (ABC) transporter, Pdr12, to prevent the accumulation of the anion S^- (Holyoak *et al.*, 1999). These processes however, may reduce energy resources significantly (Bracey *et al.*, 1998, Holyoak *et al.*, 1996, Holyoak *et al.*, 1999). Studies with benzoic acid showed that adapted *S. cerevisiae* and *Zygosaccharomyces bailii* cells reduce their permeability to benzoate (Warth, 1989, Henriques *et al.*, 1997). Changes in fatty acid composition have been reported for sorbate stressed *Z. rouxii* cells (Golden *et al.*, 1994). *Z. bailii* is able to degrade benzoate and sorbate (Mollapour & Piper, 2001) and species of *Penicillium* can decarboxylate sorbate to 1,3-pentadiene (Kinderlerer & Hatton, 1990).

Compared to yeasts, very little is known about specific weak acid resistance mechanisms in bacteria. Depending on the species, bacteria can induce several systems to counteract a drop in the internal pH when encountering low pH stress. Among others, these include proton pumps, several decarboxylases (lysine, glutamate, arginine), production of urease, arginine deiminase, chaperones (e.g. DnaK, GroELS) and sigma factor (SigB, SigM, RpoS) mediated responses (Beales, 2004, Bearson *et al.*, 1997, Cotter & Hill, 2003, Hecker & Volker, 2001,

Thackray & Moir, 2003). However, the importance of low pH stress response systems in weak acid resistance development remains unclear.

The Gram-positive bacterium *Bacillus subtilis* is one of the organisms that causes food spoilage and its growth is inhibited by sorbic acid (Eklund, 1983). This rod-shaped bacterium commonly lives in the upper layers of soil and is therefore found on crops and in food products. Thus, we investigated the time-resolved genome-wide response of *B. subtilis*, sublethally stressed with potassium sorbate (KS) using DNA microarray technology. We used the complementary methods of hierarchical clustering and T-profiler, adapted for *B. subtilis*, to analyze the data. Our results indicate that sorbic acid induces responses normally seen upon nutrient limitation. However, mild sorbic acid stress does not lead to the induction of the general stress response (GSR), sporulation or competence. *B. subtilis* likely remodels its plasma membrane, possibly to reduce the entry of sorbic acid into the cell.

2.3. Materials and Methods

2.3.1. Bacterial strains and growth conditions

All *Bacillus subtilis* strains used in this study are derivatives of the laboratory wild-type (WT) strain PB2 (*trpC2*). PB2, PB153 (*trpC2*, *sigBΔ2::cat*) and PB198 (*amyE::P_{ctc}-lacZ*) were kindly provided by C.W. Price. Mutant strains ATB002 (*ureC*), ATB008 (*sigL*) and ATB003 (*padC*) were obtained by transformation of strain PB2 with chromosomal DNA of strains SF168U (*trpC2*, *ureC::spc*) (Cruz-Ramos *et al.*, 1997), QB5505 (*trpC2*, *sigL::aphA3*) (Debarbouille *et al.*, 1991) and BS783 (*trpC2*, *padC::cat*) (Duy *et al.*, 2007), respectively. To obtain mutant strains ATB001 (*fabHB*), ATB004 (*ycsF*), ATB005 (*yhcA*), ATB006 (*yhcB*) and ATB007 (*yxkJ*), the WT strain PB2 was transformed with chromosomal DNA of strains YHFBd (*trpC2*, *fabHB::pMUTIN*), YCSFd (*trpC2*, *ycsF::pMUTIN*), YHCAd (*trpC2*, *yhcA::pMUTIN*), YHCBd (*trpC2*, *yhcB::pMUTIN*) and YXKJd (*trpC2*, *yxkJ::pMUTIN*), respectively, which were all received from the Japanese Consortium for Functional Analysis of the *B. subtilis* Genome (JAFAN, <http://bacillus.genome.ad.jp/>). Transformants were selected on LB (Luria-Bertani) agar plates containing appropriate antibiotics, after overnight incubation at 37°C. Depending on the strain, the used antibiotics were chloramphenicol (6 µg/ml), erythromycin (0.5 µg/ml), spectinomycin (100 µg/ml) or kanamycin (10 µg/ml). Isolation of chromosomal DNA was performed according to Ward and Zahler (1973) and transformations were carried out as described by Kunst and Rapoport (1995).

B. subtilis strains were cultivated in a defined minimal medium as described by Neidhardt *et al.* (1974) as modified by Hu *et al.* (1999). The medium was buffered with 80 mM 3-[N-morpholino]propanesulfonic acid (MOPS) and the pH was set to 5.9, 6.4, 7.4 or 7.8 with KOH. As carbon- and nitrogen-sources 5 mM glucose, 10 mM glutamate and 10 mM NH₄Cl were used. A 5-fold (25 mM) increase of glucose, a 10-fold (100 µM) or a 25-fold (250 µM) increase of iron was used where indicated. All strains were grown exponentially, transferred to a SpectroMax Plus microtitre plate reader (Molecular Devices Corp.) at an optical density at

600 nm (OD₆₀₀) of 0.08 (which corresponds to an OD₆₀₀ of 0.2 in a 1 cm path length spectrophotometer) and stressed with various concentrations of potassium sorbate (KS) ranging from 1.25 – 125 mM or 5 µg/ml cerulenin were indicated. Cells were further cultivated in the microtitre plate reader under rigorous shaking at 37 °C for 180 min. All conditions were tested in the microtitre plate reader at least in duplicate and biologically independent experiments were performed at least twice.

2.3.2. Assay of β -galactosidase activity

PB198 (*amyE::P_{ctc}-lacZ*) was grown exponentially in shake flasks in defined medium at pH 6.4 to an optical density at 600 nm (OD₆₀₀) of 0.2 and stressed with 3, 7, and 20 mM KS or 0.3 M NaCl. To determine the β -galactosidase activity, 1 ml samples were collected every 15 min for 1 h, frozen in liquid nitrogen and stored at -20 °C until further processing. The β -galactosidase assay was performed as described previously (Kenney & Moran, 1987). Cells were permeabilized using 0.002% SDS and 4% chloroform (final concentrations). LacZ activities were calculated as Miller units (Miller, 1972).

2.3.3. Preparation of total RNA for transcriptome analysis and real-time reverse transcriptase (RT) PCR

An exponentially growing culture of *B. subtilis* WT strain PB2 was split in two and inoculated in well-controlled batch-fermentors (500 ml working volume) to an OD₆₀₀ of 0.05. The cultures were grown at 37°C in defined medium at pH 6.4 with an aeration rate of 0.5 liters/min and vigorous stirring (200 rpm). At an OD₆₀₀ of 0.2, one culture was stressed with 3 mM KS. Samples of 20 ml were withdrawn from both the treated and control cultures at 0, 10, 20, 30, 40, and 50 min after addition of KS. Glucose levels and oxygen consumptions were obtained as described elsewhere (Alexeeva *et al.*, 2003). The cells were collected using a vacuum-filtering set-up, immediately quenched in liquid nitrogen and stored at -80 °C prior to RNA extraction. The whole procedure took no longer than 50 s. Two biologically independent experiments were performed. Total RNA was isolated as described previously (Keijser *et al.*, 2007).

2.3.4. Synthesis of labelled cDNA, hybridization, and scanning of the DNA microarrays.

Superscript II reverse transcriptase (Invitrogen) was used to synthesize labelled cDNA from total RNA samples by direct incorporation of Cy3- or Cy5-labelled dUTP into cDNA. The reaction mixture performed in First-Strand buffer contained 12 µg of total RNA, 0.5 µg of random hexamers (GE Healthcare), 400 Units of Superscript II reverse transcriptase, 10 mM dithiothreitol, 0.5 mM dATP, dCTP, dGTP and 0.2 mM dTTP (GE Healthcare), and 0.07 mM Cy3- or Cy5-dUTP (GE Healthcare). Control and sorbate treated samples were incorporated with Cy3- and Cy5-labelled dUTP, respectively. After cDNA synthesis, the RNA was hydrolyzed using 1.5 µl of 1 M NaOH for 10 min at 70°C. The pH was neutralized with 1.5 µl

of 1 M HCl and the labelled cDNA was purified by using QIAquick PCR purification spin columns (Qiagen). The efficiency of labelling was monitored spectrometrically on a Nanodrop (Isogen Life Science). The *B. subtilis* DNA microarrays were constructed as described by Keijser *et al.* (Keijser *et al.*, 2007). Each constructed array contains spots in duplicate with 4,100 gene specific 65-mer oligonucleotides, representing 4,100 of the 4,106 protein-coding genes in *B. subtilis* (as reported for the *B. subtilis* genome at <http://genolist.pasteur.fr/SubtiList/>). Hybridization and scanning was performed as described earlier (Keijser *et al.*, 2007).

2.3.5. Microarray data extraction and processing

Quantification of the hybridization signals from both Cy3- and Cy-5 channels and background subtractions was carried out with ArrayVision 6.1 software (Imaging Research Inc.). First, the pixels with density values that exceeded four median absolute deviations above the median were removed and the average of all pixels remaining in the spot was computed for each channel (the artifact-removed (ARM) density values). Second, the local background was calculated for each spot and subtracted from its ARM density value (resulting in the subtracted artifact-removed (sARM) density value). Spots with a signal-to-noise ratio (sARM divided by the standard deviation of the local background) smaller than 2.0 in both channels were excluded from further analysis. The remaining data was normalized in J-Express Pro 2.6 software (MolMine AS) using a global LOWESS (locally weighted scatterspot smoothing) algorithm (Yang *et al.*, 2002b). To avoid extreme intensity ratios, low intensity fluorescence data was floored at a value corresponding to a signal-to-noise ratio of 2.0. The data was averaged, \log_2 transformed and missing values were replaced by the average of the closest values. Genes with more than two missing values in the time-series were omitted. Since the variation in differential expression measurements depends on the fluorescent signal intensity (smaller variation at higher- and larger variation at lower fluorescence intensity levels), we applied an intensity-dependent method to identify differentially expressed genes (Yang *et al.*, 2002a). A sliding window of 50 genes was selected to calculate a Z-score from the local mean and standard deviation using the data in the R-I plot ($\log_{10}(\text{Cy5} \times \text{Cy3})$ vs. $\log_{10}(\text{Cy5}/\text{Cy3})$). Genes more than 1.96 standard deviations away from the local average ($|Z| > 1.96$) were considered differentially expressed. This corresponds to a confidence level of 95%. Genes that showed significant expression at $t = 0$ min were excluded from further analysis, unless the gene showed opposite significant expression in at least one of the other time-points. After the processing of the microarray data 3,909 genes remained for each time-point, of which 459 were found to be significantly expressed. The degree of enrichment or depletion for a specific gene group in the given significantly up- or downregulated genes was quantitatively assessed using hypergeometric distribution analysis (Motulsky, 1995). Gene groups were considered enriched or depleted when the calculated P value was below 0.01.

2.3.6. Microarray data analysis

Hierarchical clustering (Eisen *et al.*, 1998) of the significantly regulated genes was used to identify groups of genes with similar transcription profiles. In J-Express Pro 2.6 (MolMine AS) all 459 genes showing significant expression during KS treatment were hierarchically clustered using the average linkage (WPGMA) clustering method and Euclidian distance metric.

To assess the contribution of the expression of genes from specific gene classes to the total gene expression of all 3,909 genes, we used T-profiler (Boorsma *et al.*, 2005). T-profiler was adapted for the use of *B. subtilis* transcriptome data by implementing pre-defined gene groups from the following sources: the database of transcriptional regulation in *Bacillus subtilis* (DBTBS), release May 2006 (Makita *et al.*, 2004), the Kyoto Encyclopedia of Genes and Genomes database (KEGG), release May 2006 (Kanehisa *et al.*, 2006), the SubtiList database (Moszer *et al.*, 2002) and the stringently controlled genes (Eymann *et al.*, 2002). Gene groups regulated positively or negatively by a specific transcription factor were named accordingly. For transcription factors acting both as an activator and repressor, separate gene groups were made. The composition of the individual gene groups can be found in the aforementioned sources.

2.3.7. Relative quantification of gene expression using real-time reverse transcriptase PCR (RT-PCR)

For the real-time RT-PCR, RNA isolated for the DNA microarray was used to make cDNA. RT reactions were performed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Equal amounts of total RNA (5 µg) and 150 ng of random hexamers (GE Healthcare) were used in RT reactions. The amplification and detection of PCR product were performed with the 7300 real-time PCR system (Applied Biosystems). Primer Express 3.0 software (Applied Biosystems) was used to design specific primers (purchased from Isogen Life Science) for real-time PCR (see Table S1 in Appendix A). Reactions were carried out in 20 µl, consisting of 3-9 µM specific primers, 2 µl of 200-fold diluted cDNA template, and SYBR green PCR master mix (Applied Biosystems). The cycling conditions were: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. Melting curves were used to monitor specificity of the reaction. RNA of all time-points and independent experiments used in the microarray analysis were analyzed with real-time PCR in duplicate. Because the amplification of the target and reference genes was tested and found to be approximately equal (not shown), the $\Delta\Delta C_T$ method could be used to calculate relative gene expressions (Livak & Schmittgen, 2001). The expression levels of the investigated genes were determined relative to the untreated reference group. The ratios ($2^{-\Delta\Delta C_T}$) were calculated and \log_2 transformed. The *accA* gene was used as the internal control, since expression of this gene was constant in both control and stress conditions in both microarray and real-time PCR experiments.

2.3.8. Microarray accession number

Microarray data are deposited in the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE9823.

2.4. Results

2.4.1. Growth inhibition by sorbic acid can be mainly attributed to the undissociated form of the acid

Weak acids in solution are in equilibrium between their undissociated and dissociated form. To investigate whether undissociated (HS) or dissociated (S^-) sorbic acid is responsible for growth inhibition of *B. subtilis*, the pH dependence of sorbate action was tested on exponentially growing cells in a defined minimal medium. By using a defined and buffered medium, the pH remains stable and the unwanted presence of weak organic acids in undefined rich media, such as LB, is avoided. *B. subtilis* WT strain PB2 was grown exponentially in the presence of KS (0-40 mM) at pH 5.9, 6.4, 7.4 and 7.8 (Fig. 2.1A). Clearly, the reduction in growth rate is KS concentration dependent. Note that the drop in OD_{600} observed at the end of exponential phase coincides with depletion of glucose (confirmed by HPLC, our unpublished data), which may be due to a diffusion limitation of oxygen in the microtitre plate experiments. Cells grown into stationary phase in the presence of excess glucose did not show this phenomenon (our unpublished data). Similar qualitative trends of the growth curves were observed for all tested pH values. However, at higher pH, higher concentrations of KS were needed to similarly affect growth (our unpublished data). We plotted the percentage of growth inhibition (GI %) against the concentrations of S^- and HS, respectively, and observed that the curves for HS had a clear overlap, in contrast to those for S^- (Fig. 2.1B and C). This demonstrates that HS is largely responsible for the growth inhibition and its effect is pH independent. However, a closer look at the lower concentration range of HS (inset of Fig. 2.1C) reveals a pH dependence, suggesting that S^- also contributes to the growth inhibition, although to a much lesser extent. These findings are in general agreement with previous observations (Davidson, 2001, Eklund, 1983).

Noteworthy, sorbic acid stress lowers the maximally obtained OD_{600} (Fig. 2.1A), which suggests a lower yield. In order to investigate this in more detail we grew cells in well aerated batch-fermentors, stressed them with 3 mM potassium sorbate at pH 6.4, and measured the oxygen consumption and glucose levels. This treatment resulted in a 29% reduction of the growth rate. The calculated yield values on oxygen decreased 20% and on glucose 16% during 50 min of sorbic acid treatment. This suggests that sorbate lowers the energetic efficiency of respiration, which may be a consequence of a decreased proton gradient and/or cellular reprogramming. Compared to the untreated control, the glucose flux per cell calculated over this time-interval decreased with 10%, which indicates the uptake of glucose and/or its further metabolism is hampered or adjusted by the cell.

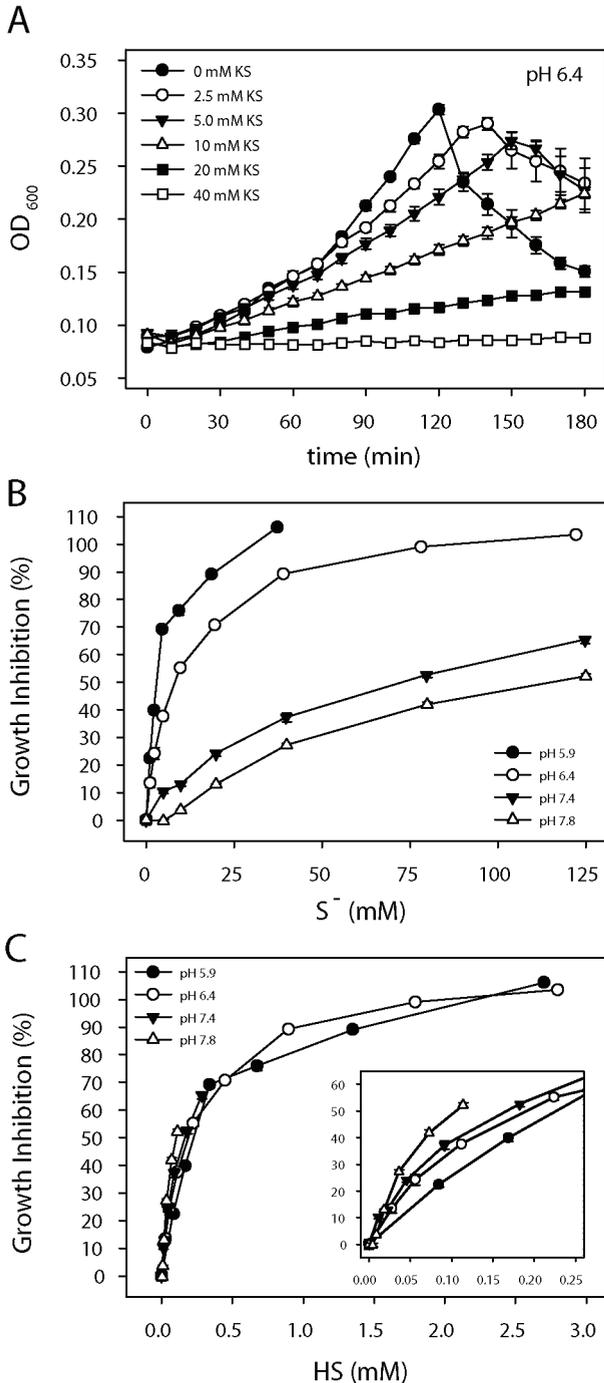


Fig. 2.1. Growth inhibition of exponentially growing *B. subtilis* by KS at various pH values. **(A)** The growth of WT PB2 in defined minimal medium at pH 6.4 was monitored in a microtitre plate reader. The closed circles indicate the growth of the control experiment (no addition of KS). Stress conditions are indicated as follows: 2.5 mM (open circles), 5 mM (closed triangles), 10 mM (open triangles), 20 mM (closed squares), and 40 mM (open squares) KS. The optical density (OD₆₀₀) was followed during 180 min. The values represent the mean of four measurements including the standard error. The percentage of growth inhibition, as compared to the control (no addition of KS), as a function of the calculated concentration of **(B)** dissociated (S⁻) and **(C)** undissociated sorbic acid (HS) molecules. The percentages of growth inhibition were calculated from the increase in optical density between 56 and 108 min after sorbate stress. The inset shows a detailed part of the lower range of the calculated HS concentration. Experiments were performed at pH 5.9 (closed circles), 6.4 (open circles), 7.4 (closed triangles), and pH 7.8 (open triangles). The values represent the mean of four measurements including the standard error.

2.4.2. Time-resolved transcriptome analysis of sorbic acid treated *B. subtilis*

To obtain a better understanding of the response of *B. subtilis* enduring sorbic acid stress, DNA microarray analysis was performed. We studied the changes in gene expression in cells exponentially grown in batch-fermentors. Samples were taken at 10, 20, 30, 40 and 50 min after exposure to 3 mM KS at pH 6.4 (GI % = 29), and compared to an untreated control. Using a fluorescent signal intensity-dependent method (see Materials and Methods) we identified a total of 459 genes (11.2% of the genome) that were differentially expressed in at least one time-point in comparison with the untreated samples (see Table S2 in Appendix A). We used real-time RT-PCR to validate the results of the microarray and selected seven transcripts representative of various temporal expression patterns observed (see Table S3 in Appendix A).

2.4.3. Microarray interpretation using hierarchical clustering and T-profiler

To analyze the data obtained from the microarray experiments we used two complementary methods: hierarchical clustering (Eisen *et al.*, 1998) to identify groups of genes (clusters) with similar transcription profiles, and T-profiler (Boorsma *et al.*, 2005) to determine significantly regulated gene groups. All 459 genes that showed significant changes were hierarchically clustered and divided into four main clusters using an arbitrary distance cut-off, indicated by the dashed blue line in the hierarchical tree (Fig. 2.2). Subdivisions of these clusters are indicated by coloured bars and letters next to each cluster. The complete hierarchical clustering, together with all gene names, their descriptions and functional categories, is available in Appendix A (Fig. S1).

T-profiler (<http://www.science.uva.nl/~boorsma/t-profiler-bacillusnew/>), developed originally for the analysis of *S. cerevisiae* (Zakrzewska *et al.*, 2005) and *Candida albicans* transcriptome data, was adapted for the analysis of genome-wide expression data of *B. subtilis* (see Material and Methods). T-profiler optimally uses all data, in contrast to hierarchical clustering where a cut-off for significance is applied. Importantly, T-profiler transforms transcriptional data of single genes into the behaviour of gene groups, reflecting biological processes in cells. All gene groups with significant *T* values in any time-point are presented in Appendix A (Tables S4 – S8).

2.4.4. Global adaptive responses to sorbic acid stress in *B. subtilis*

B. subtilis has different global adaptive responses that can be induced when it encounters stress or starvation. The GSR, regulated by sigma factor SigB, is induced by many different types of stress (e.g. glucose starvation, heat, low external pH, salt, ethanol) and provides the cell with a non-specific, multiple and preventive stress resistance (Hecker & Volker, 2001). Rather unexpectedly, we found no evidence for the induction of the GSR in our microarray analysis. Group-wise analysis using T-profiler yielded no significant values for the SigB

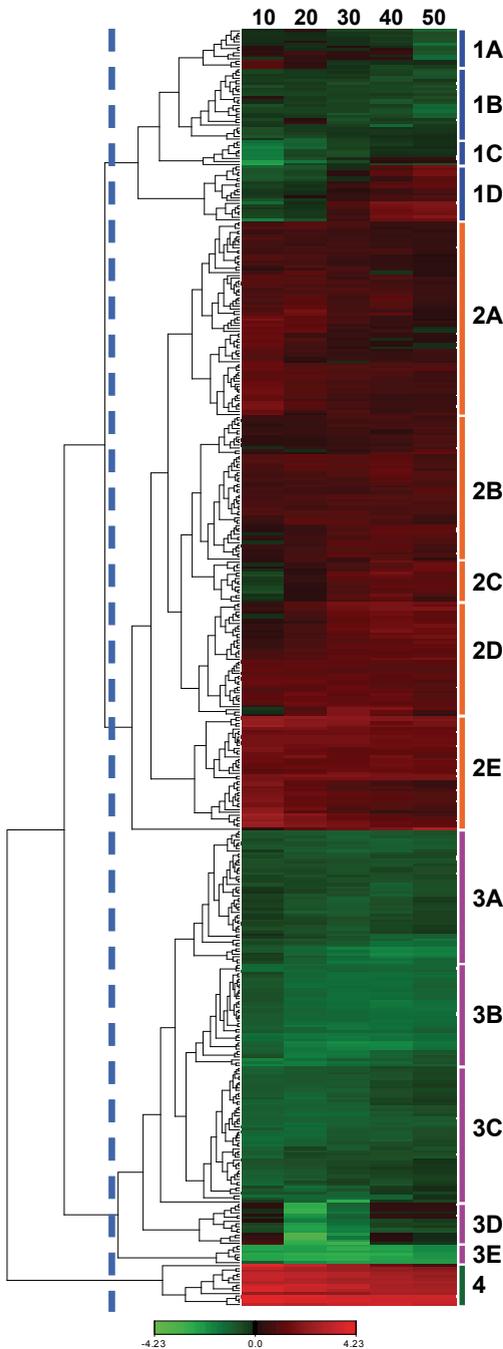


Fig. 2.2. Hierarchical clustering of significantly regulated genes. All 459 genes showing significant expression during potassium sorbate (KS) treatment were hierarchically clustered using the average linkage (WPGMA) clustering method and Euclidian distance metric (J-Express PRO 2.6, MolMine AS). Genes with similar expression are grouped in clusters. Times refer to minutes after addition of KS to exponentially growing cells. Log₂ ratios are displayed colourimetrically ranging from +4.23 to -4.23. Red indicates higher transcript levels in stressed cells than in control cells; green indicates a reduction in mRNA content. Four main clusters (using an arbitrary distance cut-off) are indicated by the dashed blue line in the hierarchical tree. Subdivisions of clusters are indicated by coloured bars and letters next to each cluster. The used log₂ ratios of each gene are the average of two independent experiments.

regulated gene group (see Fig. S2A in Appendix A), and SigB regulated genes were not overrepresented amongst the 256 upregulated genes (2 out of 95 SigB regulated genes, $P = 0.080$). In addition, the reporter strain PB198 (*amyE::P_{ctc}-lacZ*) used to monitor the induction state of the GSR, showed no induction (see Fig. S2B in Appendix A). Only very strong inhibition of growth (71%) caused by 20 mM KS resulted in increased LacZ activity. A *sigB* mutant strain showed susceptibility to KS similar to WT strain for all concentrations tested in liquid medium (Table 2.1). Additionally, long-term stress survival, as tested by spotting 10-fold dilution series of exponentially growing WT and *sigB* mutant cells on plates containing KS, revealed no difference, even at high KS concentrations (our unpublished data). We conclude that the GSR is not the key response of cells encountering sorbic acid stress.

When nutrients become limiting at the end of exponential growth and cells enter the stationary phase, the genes of the SigH regulon and the genes repressed by transition state regulator AbrB and early-stationary-phase regulator CodY are activated to adapt to the limiting conditions (Sonenshein, 2005, Strauch & Hoch, 1993). T-profiler showed a clear induction of transition-state and early-stationary-phase genes (Fig. 2.3A). Indeed, 6 out of 29 ($P = 0.0021$) genes of the SigH regulon were significantly induced (Fig. 2.2, clusters 2A, 2E and 4). Also, repression of CodY and AbrB regulated genes was relieved immediately after treatment with sorbic acid (Fig. 2.3A). This observation is further supported by the significant induction of 18 out of 42 ($P < 0.0001$) genes negatively regulated by CodY, and the induction of 15 out of 59 ($P < 0.0001$) genes repressed by AbrB, as well as the downregulation of 6 out of 20 ($P < 0.0001$) genes positively regulated by AbrB. These data indicated that the activity

Table 2.1. Susceptibility of selected mutant strains to sorbic acid stress.^a

Strain	Relevant genotype	μ (h ⁻¹) at KS of 0 mM	GI % (SD) KS (mM) of:			
			3	6	15	30
PB2	-	0.93 (0.03)	28.7 (3.0)	43.4 (1.5)	63.4 (1.8)	73.3 (1.4)
ATB001	<i>fabHB</i>	0.87 (0.04)	29.6 (5.2)	41.8 (2.7)	63.8 (1.1)	73.6 (0.3)
ATB002	<i>ureC</i>	0.97 (0.02)	31.5 (2.6)	45.1 (0.9)	61.4 (1.3)	71.9 (1.3)
ATB003	<i>padC</i>	0.97 (0.01)	19.4 (0.4)	35.2 (0.9)	63.0 (0.7)	75.3 (1.3)
ATB004	<i>ycsF</i>	0.87 (0.03)	29.5 (0.5)	41.6 (1.0)	62.5 (0.5)	72.2 (0.5)
ATB005	<i>yhcA</i>	0.99 (0.02)	17.1 (2.4)	28.0 (1.4)	63.2 (3.1)	73.7 (3.9)
ATB006	<i>yhcB</i>	0.88 (0.02)	28.0 (0.5)	40.2 (2.4)	61.8 (1.4)	70.7 (4.2)
ATB007	<i>yxkJ</i>	0.91 (0.04)	30.2 (1.9)	44.4 (1.9)	67.2 (3.7)	75.6 (2.9)
PB153	<i>sigB</i>	0.91 (0.01)	27.5 (1.7)	43.3 (0.6)	63.2 (0.5)	74.3 (0.4)
ATB008	<i>sigL</i>	0.89 (0.07)	31.0 (5.3)	41.1 (4.5)	65.7 (1.0)	75.3 (4.7)

^a KS was added to exponentially growing cultures of an OD₆₀₀ of 0.2 at pH 6.4. Specific growth rates (μ) were calculated from the increase in optical density between 56 and 108 min after sorbate stress. Growth inhibition percentages (GI %) were calculated from the obtained growth rates and are relative to the specific growth rate in the control condition (no addition of KS). All conditions were tested least in duplicate and biologically independent experiments were performed at least twice. The standard deviation is shown in parentheses.

of AbrB decreased transiently in the first 20 min after sorbate stress. To corroborate the derepression of genes negatively regulated by CodY we analyzed and confirmed by real-time RT-PCR the induction of gene *ybgE* (see Table S3 in Appendix A). This gene is known to be only regulated by CodY (Molle *et al.*, 2003). The induction of transition state and early-stationary-phase genes suggests that sorbic acid stressed cells respond as if they experience a nutrient limitation.

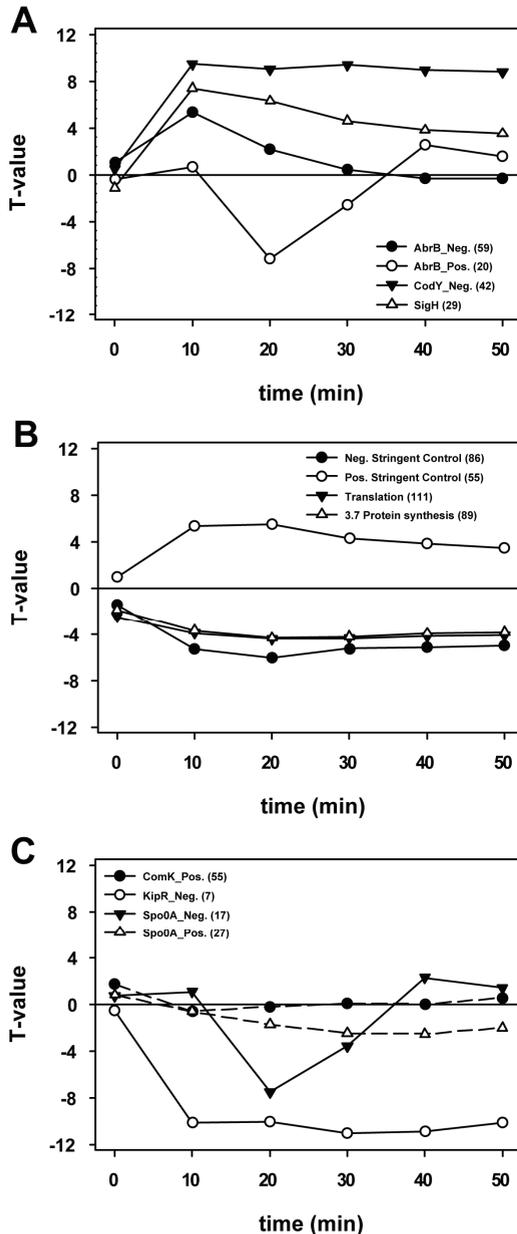


Fig. 2.3. Global adaptive responses to sorbic acid stress in *B. subtilis* as revealed by T-profiler. **(A)** Induction of transition state and early-stationary-phase gene groups. The *T* values of gene groups regulated negatively (closed circles) and positively (open circles) by AbrB, negatively by CodY (closed triangles) and by SigH (open triangles) are shown. **(B)** Induction of the stringent response. The *T* values of gene groups negatively (closed circles) and positively (open circles) controlled by RelA, involved in translation (closed triangles) and proteins synthesis (open triangles) are shown. **(C)** No induction of competence or sporulation. The *T* values of gene groups regulated positively by ComK (closed circles), negatively by KipR (open circles) and negatively (closed triangles) and positively (open triangles) by Spo0A are shown. A distinction is made between groups of genes that are positively or negatively regulated by the transcription factor mentioned (indicated by Pos. or Neg.). The number of ORFs within each group present in the microarray experiments is shown in parentheses. The *T* values presented are calculated on the basis of two biologically independent experiments. Shown are gene groups that have at least one significant *T* value ($E < 0.05$) in the time-course analyzed. The gene groups regulated negatively by ComK and positively by Spo0A (dashed lines) did not show significant *T* values in the time-course analyzed.

In amino acid-, glucose- or oxygen- limited cells, the (RelA mediated) stringent response helps to prevent the waste of scarce nutrients (Braeken *et al.*, 2006, Eymann *et al.*, 2002). Hierarchical clustering revealed only 8 out of 55 ($P = 0.017$) RelA-dependent positive stringent control genes significantly induced and 4 out of 86 ($P = 0.79$) RelA-dependent negative stringent control genes significantly repressed. However, upon considering all genes, as is done in T-profiler, significant T values for both (RelA-dependent) positive and negative stringent control gene groups were found (Fig. 2.3B). The significant downregulation of gene groups involved in translation and protein synthesis (Fig. 2.3B), as well as purine and pyrimidine synthesis (Tables S4, S6 and S7 in Appendix A), further reflects the induction of the stringent response and the observed reduction in growth.

Nutrient limitation can trigger the onset of sporulation and the development of competence (Dubnau, 1991, Sonenshein, 2000). However, we found no concerted induction of genes controlled by the competence key regulator ComK (Fig. 2.3C), of other known regulators involved in competence, or of the functional category transformation/competence (our unpublished data). Sporulation was also not induced by sorbic acid. We found a clear downregulation of the KipR regulated genes (Fig. 2.3C and cluster 3E in Fig. 2.2), which may indicate the release of the 'brake' on the sporulation regulatory cascade. KipR is the negative regulator of the *ycsFGI-kiplAR-ycsK* operon (Wang *et al.*, 1997). Inactivation of *ycsF*, the first gene of this operon, did not lead to an altered susceptibility towards sorbic acid (Table 2.1). Kipl is an inhibitor of KinA, the primary kinase in the phosphorelay necessary for the phosphorylation of the key transcription factor Spo0A that regulates the initiation of sporulation (Sonenshein, 2000). However, no induction of genes positively regulated by the sporulation master regulator Spo0A was observed (Fig. 2.3C). There is a brief downregulation of genes repressed by Spo0A (8 out of 17, $P < 0.0001$), but this can be fully explained by the downregulation of the arginine biosynthesis genes controlled by AbrB and arginine metabolism regulator AhrC (Fig. 2.2, cluster 3D and Table S4 in Appendix A). Furthermore, we did not observe induction of other sporulation gene groups regulated by SpoIIID, SpoVT, or belonging to the SigE, F, G and K regulon (our unpublished data). Compared to the untreated control, no increased spore counts were detected in sorbic acid stressed cultures (our unpublished data). Concluding, we see no signs of the development of competence or induction of sporulation.

2.4.5. Responses to counteract intracellular acidification

One of the most prominent effects of weak acids on the microbial cell may be the cytosolic acidification. We discovered a brief but significant induction of the genes coding for class I heat-shock proteins GroES and GroEL (Fig. 2.2, clusters 1A and 2A). Chaperones are known to play a role in the maintenance of protein folding at lowered pH, and the induction of *groEL* upon acid stress has been described in *Lactobacilli*, *Streptococcus mutans*, *Clostridium perfringens*, and *Listeria monocytogenes* (Cotter & Hill, 2003).

We investigated cellular functions, which may counteract the putative pH drop and maintain pH homeostasis. We found no significant induction of ATPases and components of the respiratory chain. However, the capacity of the cell's ATPases and respiratory chain may well be sufficient to maintain pH homeostasis without regulation on the transcription level. We did observe altered expression of 3 out of 6 genes ($P < 0.0001$) belonging to the Gene Ontology group involved in the regulation of pH (GO:0006885). The following genes were significantly downregulated: *nhaC* (Na^+/H^+ antiporter), *yjbQ* (similar to Na^+/H^+ antiporter), and *yuiF* (similar to hypothetical proteins). A highly upregulated gene coding for a proton symporter that transports both citrate and malate was *yxkJ* (Krom *et al.*, 2001). The over 12-fold induction of this gene (Fig. 2.2, cluster 4) might indicate its involvement in sorbic acid tolerance. A strain mutated for *yxkJ* was however not more sensitive to sorbic acid than the WT (Table 2.1). Long-term stress survival on plates containing KS did not reveal sensitivity either (our unpublished data).

Interestingly, we found all three genes of the *ureABC* operon, which code for the structural components of urease (Cruz-Ramos *et al.*, 1997), in cluster 4 with the most upregulated genes (Fig. 2.2). Regulation of this operon is complex, as it is regulated by PucR, TnrA, CodY, and GlnR and expression is dependent on SigA and SigH (Brandenburg *et al.*, 2002, Wray *et al.*, 1997). In addition, induction of *ureA* is also RelA dependent (Eymann *et al.*, 2002). Thus, the induction of the *ureABC* operon may be explained by the derepression of CodY regulated genes and induction of the stringent response (Fig. 2.3A and B). The upregulation of this operon might be involved in counteracting the possible acidification of the cytosol through the production of basic ammonia, as in some bacterial pathogens, ureases are known to facilitate survival in acidic environments (Collins & D'Orazio, 1993, Mobley *et al.*, 1995). However, a urease mutant was not KS sensitive in liquid (Table 2.1) or solid cultures (our unpublished data).

Although sorbic acid is not a phenolic acid, we surprisingly found strong upregulation of the phenolic acid decarboxylase, *padC* (Cavin *et al.*, 1998) in cluster 4 (Fig. 2.2). The genes with unknown function *yveF* and *yveG*, which lie upstream of *padC* in the genome, were also strongly induced and present in this cluster, which may indicate the co-regulation of these three genes. The inactivation of *padC* did not lead to KS sensitivity. Surprisingly, the mutant strain showed a clear resistant phenotype at low KS concentrations both in liquid cultures (Table 2.1) and on solid cultures (our unpublished data).

2.4.6. Responses to cope with a decreased proton gradient

The proposed influx of protons mediated by the diffusion of the weak acid over the cell membrane may lead to a decreased proton motive force. Also, the expulsion of protons to maintain pH homeostasis by ATPases and/or the respiratory chain could lead to a higher demand on energy resources, which should have a negative effect on the yield. The latter was indeed measured as reported above.

Interestingly, we observed major changes in genes involved in carbon metabolism upon sorbic acid stress (Fig. 2.4A). Besides the induction of carbohydrate metabolism, as well as fructose and mannose metabolism gene groups, we found a strong upregulation of genes negatively regulated by the carbon catabolite control protein CcpA after 20 min of sorbic acid exposure. Carbon catabolite derepression normally occurs when the preferred carbon source (glucose, fructose, and mannose) is depleted (Deutscher *et al.*, 2002). However, glucose was still present in the medium and consumed by the cells during sorbic acid treatment (confirmed by HPLC, our unpublished data), although the glucose flux per cell decreased by 10%. Noticeably, a fivefold (25 mM) increase in the glucose concentration in the medium did not alter the growth-inhibitory effect of sorbic acid (Table 2.2).

The expression of genes dependent on SigL (involved in alternative carbon and nitrogen metabolism) also produced significant T values after 20 min of sorbic acid exposure (Fig. 2.4A). The induction of the SigL-dependent *levD* gene was confirmed by real-time RT-PCR (see Table S3 in Appendix A). However, the inactivation of the *sigL* gene did not change the susceptibility to sorbic acid compared to the WT (Table 2.1).

Genes of the tricarboxylic acid (TCA) cycle were also induced gradually during sorbic acid stress (Fig. 2.4A). The TCA cycle can be fed by additional acetyl-CoA produced from the breakdown of the catabolic products acetate and acetoin. Indeed, we found a significant

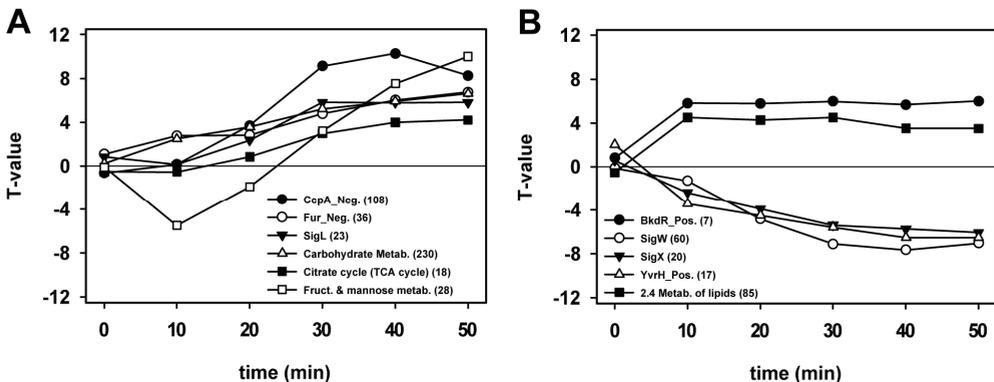


Fig. 2.4. T-profiler analysis of the transcriptional response to sorbic acid stress reveals induction of possible specific adaptation mechanisms. **(A)** Responses indicating adaptation mechanisms against uncoupling of the proton gradient, energy and nutrient limitation. The T values of gene groups regulated negatively by CcpA (closed circles) and Fur (open circles), SigL (closed triangles), Carbohydrate metabolism (open triangles), Citrate cycle (TCA cycle) (closed squares), and Fructose and mannose metabolism (open squares) are shown. **(B)** Responses indicating remodelling of the cell envelope. T values of gene groups regulated positively by BkdR (closed circles), SigW (open circles), SigX (closed triangles), positively by YvrH (open triangles), and involved in the metabolism of lipids (closed squares) are shown. A distinction is made between groups of genes that are positively or negatively regulated by the transcription factor mentioned (indicated by Pos. or Neg.). The number of ORFs within each group present in the microarray experiments is shown in parentheses. The T values presented are calculated on the basis of two biologically independent experiments. Shown are gene groups that have at least one significant T value ($E < 0.05$) in the time-course analyzed.

induction of the acetyl-CoA synthetase *acsA*, which is responsible for the degradation of acetate (Grundy *et al.*, 1993) and *acuABC*, which is involved in the activation of *AcsA* (Gardner *et al.*, 2006). Also the genes *acoA* and *acoR*, involved in the breakdown of acetoin (Ali *et al.*, 2001, Huang *et al.*, 1999), were significantly upregulated.

In addition to these carbon metabolism-related gene functions, we observed a short downregulation followed by a strong upregulation of the gene group regulated by the central iron regulatory protein Fur (ferric uptake repressor) (Fig. 2.4A). This transient profile was also clearly seen in clusters 1D and 2C of the hierarchical clustering, containing many Fur-regulated genes (Fig. 2.2). In total, we found 15 out of 36 ($P < 0.0001$) Fur-regulated genes significantly expressed in our analysis with similar transient expression profiles. The Fur regulon is normally derepressed under iron-limiting or anaerobic conditions (Moore & Helmann, 2005, Ye *et al.*, 2000). We tested whether increased iron concentrations in the medium would result in an increase in the stress tolerance against sorbic acid. A 10-fold (100 μM) or 25-fold (250 μM) increase of iron in the medium could not decrease the growth inhibition by sorbic acid (Table 2.2). These combined observations suggest that although nutrients in the cell's environment are not limiting, the cells respond as if nutrients are scarce.

2.4.7. Sorbic acid influences the biogenesis of the cell envelope

Since HS can dissolve in the cell membrane and is thought to affect membrane integrity, we expected to see a membrane-remodelling response or an adaptation of the cell envelope to limit HS entry. Functions associated with the cell surface or transport are controlled by extracytoplasmic function sigma factors, like SigM, SigW and SigX (Helmann, 2002). Indeed, the gene groups regulated by extracytoplasmic function sigma factors SigW and SigX showed altered expression upon sorbic acid treatment (Fig. 2.4B). We also detected a similar repression pattern for genes regulated by YvrH (Fig. 2.4B). YvrH is involved in the maintenance of the cell surface state through transcriptional regulation and controls genes containing a promoter site for SigX, and *sigX* itself (Serizawa *et al.*, 2005). The repression of SigW- and SigX-regulated genes upon sorbic acid stress suggests their expression under control conditions. This finding was corroborated by the absolute fluorescence levels.

Table 2.2. Growth medium supplemented with either additional glucose or iron does not increase tolerance towards sorbic acid stress.^a

Glucose addition in medium (5 mM)	FeSO ₄ addition in medium (10 μM)	GI % ^b
1x	1x	28.7 (3.0)
5x	1x	30.0 (0.2)
1x	10x	31.2 (0.4)
1x	25x	32.7 (2.3)

^a See Materials and Methods for the standard composition of the medium.

^b 3 mM potassium sorbate (KS) was added to exponentially growing cultures of an OD₆₀₀ of 0.2 at pH 6.4. Growth inhibition percentages (GI %) were calculated from the obtained growth rates between 56 and 108 min after addition of KS and are relative to the specific growth rate in the control condition (no addition of KS). All conditions were tested at least in duplicate and biologically independent experiments were performed at least twice. The standard deviation is shown in parentheses.

Although SigW and SigX are activated upon cell wall- and membrane-perturbing stresses we did not have any indication that our control cells suffered stress. This was supported by a μ_{\max} of 0.93, no induction of the GSR, and no formation of heat resistant spores in the control culture (our unpublished results).

T-profiler analysis showed a strong induction of genes involved in the metabolism of lipids directly after sorbic acid exposure (Fig. 2.4B). Most fatty acid biosynthesis (*fab*) genes, negatively regulated by FapR (YlpC) (Schujman *et al.*, 2003), were significantly upregulated. The *fab* initiation genes *fabHB* and *fabD* and elongation genes *fabF*, *fabG* and *fabI* (Fig. 2.2, clusters 2A, 2E, and 4) showed significant induction with similar expression profiles. The strong upregulation (19-fold) of *fabHB* was confirmed using RT-PCR (Table S3 in Appendix A). In addition, we observed the significant induction of genes regulated by BkdR in both T-profiler analysis (Fig. 2.4B) and hierarchical clustering (Fig. 2.2, clusters 2D and E). BkdR is the activator of the *bkd* operon, consisting of seven genes involved in the synthesis of precursor molecules for branched-chain fatty acids (Debarbouille *et al.*, 1999). The induction of both the *fab* genes and the *bkd* operon in sorbic acid-treated cells will likely increase the number of long-chain and branched-chain fatty acids in the membrane (de Mendoza *et al.*, 2002, Schujman *et al.*, 2003).

We investigated the role of fatty acid synthesis in the resistance to sorbic acid stress by growing a β -ketoacyl-acyl carrier protein (ACP) synthase III mutant in the presence of sorbic acid. Strain ATB001 (*fabHB*) did not reveal a sensitive phenotype (Table 2.1). The antibiotic cerulenin inhibits the β -ketoacyl-ACP synthases (FabHA, FabHB and FabF) of the fatty acid chain elongation step (D'Agnolo *et al.*, 1973). In addition, cerulenin induces the FapR regulon (Schujman *et al.*, 2001). Remarkably, the simultaneous addition of sorbic acid and cerulenin to exponentially growing WT cells significantly decreased the inhibitory effect of the antibiotic compared to cultures treated with cerulenin alone (Fig. 2.5). This result indicates that the addition of KS may lead to a membrane adaptation that alters the cell's sensitivity to cerulenin.

2.4.8. Possible extrusion of the anion

S. cerevisiae uses the pump Pdr12 to extrude the sorbate anion to prevent accumulation (Holyoak *et al.*, 1999). Interestingly, we found the gene *yhcA* to be highly upregulated in the presence of KS (Fig. 2.2, cluster 4). YhcA is a multidrug resistance transporter homologue of the major facilitator superfamily. To investigate the role of this gene in sorbate stress tolerance, we tested mutant strain ATB005 (*yhcA*) for sorbic acid sensitivity. Surprisingly, the *yhcA* mutant strain showed clear sorbate resistance, especially at low KS concentrations in liquid medium (Table 2.1) and on solid cultures (our unpublished data). *yhcB* (similar to the *trp* repressor binding protein) is located downstream of *yhcA* in the genome. This gene also showed strong upregulation (Fig. 2.2, cluster 4) and was tested for sorbate stress tolerance.

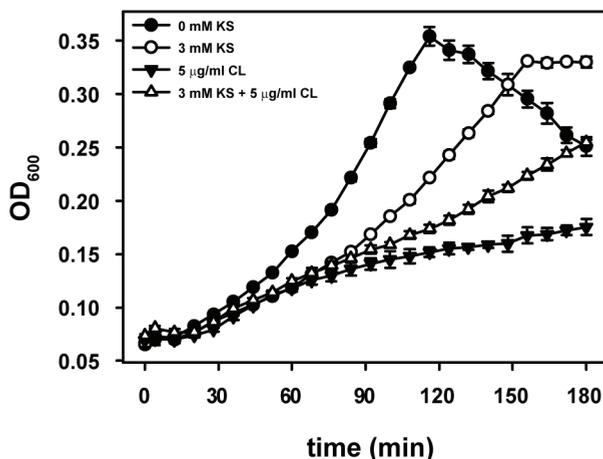


Fig. 2.5. Sorbic acid stress may lead to a membrane adaptation which renders the cells resistance to cerulenin. Exponentially growing WT PB2 cells were grown without (closed circles) or with the addition of 3 mM potassium sorbate (KS) (open circles), 5 µg/ml cerulenin (CL) (closed triangles), and 3 mM KS + 5 µg/ml CL (open triangles). The optical density (OD_{600}) was followed during 180 min. The values represent the mean of four measurements, including the standard deviation.

Mutant strain ATB006 (*yhcB*) showed no altered sorbic acid susceptibility (Table 2.1). These data illustrate that the resistance of the *yhcA* mutant strain is caused by the inactivation of *yhcA* itself and not by affected downstream genes.

2.5. Discussion

Our study shows that mainly the undissociated form of sorbic acid (HS) is responsible for the inhibition of growth of *B. subtilis* (Fig. 2.1). These data support the model that suggests that the neutral form of the acid enters the cell, where it dissociates, possibly acidifying the cytosol and likely contributing to its inhibitory effect (Beales, 2004, Davidson, 2001). Maintaining the internal pH is of crucial importance for the proper functioning of the cell. We observed several responses that may counteract the possible intracellular acidification caused by the dissociation of HS (e.g. the induction of the urease operon and the phenolic acid decarboxylase *padC*). However, thus far, we have no indication that these responses are functional in HS tolerance. Besides possible acidification of the cytosol, the entry of protons will lower the proton gradient. Consequently, this will reduce the cell's efficiency to produce ATP, which is supported by the observed lower yields on glucose and oxygen upon sorbic acid stress.

The transcriptome analysis clearly revealed that cells stressed with sorbic acid respond as if they encounter nutrient limitation. We observed a clear induction of transition-state and early-stationary-phase genes regulated by AbrB, SigH, and CodY as well as an induction of the stringent response, mediated by RelA (Fig. 2.3). The derepression of the carbon

catabolite control genes, regulated by CcpA, major changes in carbon metabolism and the induction of the Fur regulon further suggest that, even though glucose is still abundant in the medium, the cells experience nutrient limitation(s) (Fig. 2.4A). This may be caused by effects of the acid on glucose uptake systems, but may also be the result of an altered intracellular environment and effects on enzymes involved in the subsequent glucose-phosphate catabolism. This possibility is supported by the decreased glucose flux seen in sorbic acid-treated cells. Future studies should aim at (short-term) measurements of intracellular metabolites and oxygen consumption in the absence and presence of sorbic acid. The addition of excess glucose or iron to the medium did not change sorbate susceptibility (Table 2.2), which seems to point out that limitations in the medium are not the cause of the nutrient limitation responses of the cell.

Sorbic acid did not induce sporulation, competence (Fig. 2.3C) or the GSR (see Fig. S2 in Appendix A). We conclude that the limiting conditions caused by 3 mM sorbic acid are not severe enough to switch on sporulation, competence or the GSR, or that other factors inhibit these adaptation pathways. Zhang and Haldenwang (2005) recently showed that the induction of the GSR by nutritional stress is preceded by a drop in ATP levels. The absence of a GSR induction upon (mild) sorbic acid stress might indicate that ATP levels are not severely affected.

The observed active state of the SigW and SigX regulon in control *B. subtilis* cells grown in defined (MOPS based) medium is somehow surprising. Note that we opted for the use of an established *Bacillus* medium (see e.g. Hu *et al.*, 1999, Wray & Fisher, 2007)) and noted no signs of stress (see Results). The observed downregulation of SigW and SigX regulated genes upon sorbic acid stress may lead to a blockage of cell envelope remodelling and, consequently altered cell envelope composition.

Immediately after sorbic acid exposure a clear activation of genes involved in the metabolism of lipids was observed (Fig. 2.4B). The induction of both the *fab* genes and the *bkd* operon will likely increase the number of long-chain- and branched-chain- fatty acids in the membrane (de Mendoza *et al.*, 2002, Schujman *et al.*, 2003). An elongation of fatty acids in the membrane might lower the diffusion rate of sorbic acid into the cell. The simultaneous addition of more branched-chain fatty acids into the membrane through the action of the *bkd* operon will likely preserve fluidity. The inactivation of *fabHB* did not result in a sensitive phenotype for sorbic acid (Table 2.1). Noticeably, since *fabHA* codes for a β -ketoacyl-ACP synthase III as well, this gene is likely to take over the function of *fabHB* in the mutant strain tested. Recently, Thomaidis *et al.* (Thomaidis *et al.*, 2007) illustrated that the *fabHA fabHB* double mutant is nonviable. The combined treatment of cultures with both KS and cerulenin, the inhibitor of the β -ketoacyl-ACP synthases, significantly lowered the inhibitory effect of cerulenin alone (Fig. 2.5), supporting the inferred remodelling of the membrane in response to sorbic acid. Alteration of the plasma membrane composition upon sorbic acid stress in *Z. rouxii* was described previously (Golden *et al.*, 1994). In addition, *S. cerevisiae* and *Z. bailii*

cells adapted to benzoic acid have a reduced permeability to benzoate (Warth, 1989, Henriques *et al.*, 1997). We suggest that in *B. subtilis* the observed induction of the *fab* genes and *bkd* operon is a direct response to sorbic acid that will likely alter the membrane composition such that the diffusion rate of the organic molecule over the membrane is lowered.

Accumulation of the anion may also cause harm to the cell (Davidson, 2001, Hirshfield *et al.*, 2003). The strong upregulation of *yhcA*, encoding a major facilitator superfamily multidrug resistance transporter homologue, suggested a potential anion extrusion mechanism. The inactivation of *yhcA* however, revealed a clear resistant phenotype upon stress with low concentrations of KS in defined minimal liquid medium (Table 2.1) and on solid cultures (our unpublished data) under our test conditions. In theory, the extrusion of S^- by YhcA may create a futile cycle and deplete cellular energy pools, since the anion can reassociate in the extracellular environment with a proton and diffuse back into the cell, thereby reducing the proton gradient further. If such futile cycling indeed occurs, it may significantly contribute to the observed resistance of the *yhcA* mutant strain when tested in defined minimal medium. Alternative explanations, in which the YhcA protein would be the site at which sorbic acid enters the cells inadvertently and its deletion would thus lower the sorbic acid sensitivity of the cells, are also possible. Such events would be analogous to the recently reported acetic acid stress resistance mechanisms in yeast involving the Fps1 aquaglyceroporin protein (Mollapour & Piper, 2007). Interestingly, we have preliminary data showing that in rich medium the *yhcA* mutant strain is more sensitive to KS on solid cultures (our unpublished data). Future studies should address the function of YhcA and its relationship to the differences observed when minimal or rich medium is used.

In conclusion, sorbic acid induces responses normally seen upon nutrient limitation. Therefore, we suggest that the entry of the protonated acid and the subsequent lowering of the proton gradient increase the demand for energy. The cells do not (and likely cannot) increase the uptake rate of glucose and consequently experience nutrient limitation. The upregulation of the TCA-cycle and the utilization of acetate and acetoin may provide sufficient energy to maintain growth but at a lower rate. Finally, the plasma membrane is remodelled, likely in an attempt to reduce the entry of sorbic acid into the cell. Whether the observed responses in *B. subtilis* upon sorbic acid stress are representative of weak acids in general, remains to be elucidated.

2.6. Acknowledgements

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