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## Comparative physiological and transcriptional analysis of weak organic acid stress in *Bacillus subtilis*



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### ABSTRACT

The advent of 'omics' techniques bears significant potential for the assessment of the microbiological stability of foods. This requires the integration of molecular data with their implication for cellular physiology. Here we performed a comparative physiological and transcriptional analysis of *Bacillus subtilis* stressed with three different weak organic acids: the commonly used food preservatives sorbic- and acetic-acid, plus the well-known uncoupler carbonyl cyanide-*m*-chlorophenyl hydrazine (CCCP). The concentration of each compound needed to cause a similar reduction of the growth rate negatively correlated with their membrane solubility, and positively with the concentration of undissociated acid. Intracellular acidification was demonstrated by expressing a pH-sensitive GFP derivative. The largest drop in intracellular pH was observed in CCCP-stressed cells and was accompanied by the transcriptional induction of the general stress response (GSR) and SigM regulon, responses known to be induced by acidification. The GSR was induced by acetate, but not by sorbate in mildly-stressed cells. Microarray analysis further revealed that all three acids activate transcriptional programs normally seen upon nutrient limitation and cause diverse responses indicative of an adaptation of the cell envelope. Based on the responses observed and the utilized pH measurements, the inhibitory effect of sorbic acid seems to be more focused on the cell membrane than that of acetic acid or CCCP.

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### 1. Introduction

In the food industry there is a notion that the novel 'omics' data will allow processors to come to a more mechanism based risk assessment of food preservation regimes (Brul et al., 2012). Hence such approaches will allow us to come more close to the preservation cliff edge (Rantsiou et al., 2011), thus benefitting food quality whilst ensuring at all times the desired microbiological food stability (McMeekin et al., 2010). Over-processing can be avoided and the use of preservative agents minimized. 'Omics' studies at the

transcript level allow for genome wide assessment of the way in which microorganisms perceive the environment, including the presence of food preservatives such as weak organic acids.

Weak organic acids, such as sorbic- and acetic- acid, are well established antimicrobial agents utilized by the food industry. The acid, or its anionic salt, is used in a variety of foods and beverages as preservatives to inhibit the growth of bacteria, yeasts, and moulds (Beales, 2004; Brul and Coote, 1999; Davidson, 2001; Ter Beek and Brul, 2010). The antimicrobial activity of this class of food preservatives depends on the  $pK_a$  value of the acid and the pH of the environment. In solution, the compound exists in equilibrium between the dissociated and the undissociated state. The latter form is more lipid permeable than the anion, and is therefore able to diffuse into the cell, where a new equilibrium is formed. The resulting release of protons lowers the proton gradient and, depending on the buffering capacity of the cell, may cause lowering of the intracellular pH ( $pH_i$ ). Lowering the proton gradient and the possible acidification of the cytosol are thought to be the main modes of action of weak organic acids (Beales, 2004; Brul and

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Coote, 1999; Davidson, 2001; Ter Beek and Brul, 2010). Furthermore, the released anion is considered to be responsible for a rise in osmolarity and to be deleterious to cytosolic enzymes (Russell, 1992; York and Vaughn, 1964). Recently, Abee and co-workers have shown the formation of reactive oxygen species (ROS) in weak organic acid-stressed *Bacillus cereus* cells (reviewed in Mols and Abee, 2011). In addition, organic acids are thought to interfere with the cell wall, the cytosolic membrane and membrane proteins, and consequently, influence the transport of nutrients (Hirshfield et al., 2003; Sheu and Freese, 1972; Stratford et al., 2009; Ter Beek and Brul, 2010). Although the general modes of action are established, differences between the effectiveness of weak organic acids are observed. For instance, the  $pK_a$  values of sorbic and acetic acid are similar (4.76), however more acetic acid is needed to obtain similar growth-inhibitory effects (among others: Heavin et al., 2009; Lambert and Bidlas, 2007).

Depending on the microorganism challenged and the (weak) acid used, various resistance mechanisms have been described. Bacteria can induce several systems to counteract a drop in the  $pH_i$  when encountering (extreme) low extracellular pH ( $pH_{ex}$ ) stress. The general stress response (GSR) (SigB and RpoS-mediated in Gram-positive and Gram-negative bacteria, respectively) is induced upon low pH stress (Dodd and Aldsworth, 2002; Hecker et al., 2007). The SigM regulon, involved in maintaining membrane and cell wall integrity, is also induced in *Bacillus subtilis* at low  $pH_{ex}$  (Thackray and Moir, 2003). Furthermore, the induction of proton pumps, chaperones, superoxide dismutases, and the production of basic compounds by urease, decarboxylases, and deiminase are known factors contributing to (weak) acid tolerance (Bearson et al., 1997; Cotter and Hill, 2003; Kitko et al., 2009; Mols and Abee, 2011; Piper, 1999; Wilks et al., 2009).

In this study we set out to compare commonly used weak organic acid food preservatives in their molecular effects on spore forming-, spoilage bacteria. Sorbic acid and acetic acid were chosen for this analysis and compared to a classical chemical agent and weak acid that uncouples metabolism from growth: carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP). Since *B. subtilis* is well-studied, genetically accessible, and also a relevant food spoilage bacterium (Oomes et al., 2007), we selected this model microorganism for our research. Here we report the thorough comparison of the time-resolved transcriptional responses of *B. subtilis* subjected to mild potassium acetate (KAc) and CCCP stress, and the previously by us published data on potassium sorbate (KS) stress (Ter Beek et al., 2008).

## 2. Materials and methods

### 2.1. Bacterial strains, growth and stress conditions

*B. subtilis* strains were cultivated in a defined minimal medium as described previously 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  $NH_4Cl$  were used. The medium was supplemented with 5  $\mu g/ml$  kanamycin and isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, when required. *Escherichia coli* strains were grown in Luria–Bertani (LB) medium at 37 °C. When appropriate, ampicillin (100  $\mu g/ml$ ) or kanamycin (50  $\mu g/ml$ ) was used as antibiotic.

168 wild-type (WT) *B. subtilis* strain PB2 (*trp2C*) was grown exponentially in shake-flasks, transferred to a SpectraMax Plus microtiter plate reader (Molecular Devices Corp.) at an optical density at 600 nm ( $OD_{600}$ ) of 0.08 (which corresponds to an  $OD_{600}$  of 0.2 in a 1 cm path length spectrophotometer) and stressed with various concentrations of KAc (5–500 mM) or CCCP (0.25–10  $\mu M$ ).

Cells were further cultivated in the microtiter plate reader under rigorous shaking at 37 °C for 180 min. The percentages of growth inhibition (GI %) were calculated from the increase in the optical density between 50 and 120 min of the control (no addition of stress) and the stressed culture. Minimal inhibitory concentration (MIC) values were estimated using the following operational definition: the lowest concentration of compound that reduced the growth to an  $OD_{600}$  after 180 min incubation of 2-fold the starting  $OD_{600}$  or less (Schoormans et al., 2009). All conditions were tested in the microtiter plate reader at least in triplicate and biologically independent experiments were performed at least twice.

### 2.2. Construction of pDG148-pHluorin and transformation to *B. subtilis*

Standard molecular genetics techniques were used as described by Sambrook et al. (1989). Restriction enzymes, *Pfu* DNA polymerase and T4 DNA ligase were obtained from Fermentas. Plasmids were isolated from *E. coli* using a QIAprep Spin Miniprep Kit (Qiagen) and DNA fragments were isolated from agarose gels with a QIAEX II Gel Extraction Kit (Qiagen).

The ratiometric pHluorin gene (GenBank accession no. AF058694) was PCR amplified using primers pHBsF (5'-AAG CTT AAG GAG GAA GCA GGT ATG AGT AAA GGA GAA GAA C-3') and pHBsR (5'-C GTC GAC TTA TTT GTA TAG TTC ATC CAT GCC ATG TG-3') (Isogen Life Science) on pYES2-P<sub>ACT1</sub>-pHluorin (Orij et al., 2009) introducing *Hind*III and *Sall* restriction sites as well as a ribosome binding site. The pYES2-P<sub>ACT1</sub>-pHluorin plasmid contains the gene coding for a functional ratiometric pHluorin without the L220F mutation described in Miesenböck et al. (1998), as well as a start and stop codon (Orij et al., 2009). The resulting 745-bp PCR product was cloned into the pCR-Blunt II-TOPO vector and transformed to competent *E. coli* TOP10 cells using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). DNA sequence analysis (BaseClear) confirmed the correct DNA sequence (data not shown). Next, the TOPO vector was digested with *Hind*III and *Sall* and the fragment containing ratiometric pHluorin gene was inserted into the *Hind*III and *Sall*-digested pDG148 plasmid creating pDG148-pHluorin. Finally, both the empty and pHluorin containing pDG148 plasmids were transformed to competent 168 WT *B. subtilis* strain 1A700 (*trp2C*) cells as described previously (Kunst and Rapoport, 1995) creating strains ATB031 and ATB032, respectively.

### 2.3. Calibration and measurement of $pH_i$

*B. subtilis* strains ATB031 and ATB032 were grown exponentially in shake-flasks in defined minimal medium of pH 6.4 containing 5  $\mu g/ml$  kanamycin and 25 mM glucose. At an  $OD_{600}$  of 0.2 the production of ratiometric pHluorin was induced for 2.5 h by the addition of 1 mM IPTG. The cells were maintained in the exponential phase by diluting the cultures in fresh medium containing 1 mM IPTG when necessary, such that an  $OD_{600}$  of 1 was not exceeded. At an  $OD_{600}$  of 0.4 the cells were centrifuged and resuspended in buffers with pH values ranging from 5.6 to 8.2 prepared from 0.1 M citric acid and 0.2 M  $K_2HPO_4$ . The  $pH_{ex}$  and  $pH_i$  were equilibrated by the addition of 1  $\mu M$  valinomycin and 1  $\mu M$  nigericin (Breeuwer et al., 1996). The fluorescence was measured in a SpectraMax Gemini XS microtiter plate spectrofluorometer (Molecular Devices Corp.) and the ratio of emission intensity at 512 nm resulting from excitation at 390 and 470 nm was calculated after background subtraction as described previously (Orij et al., 2009). The strain containing the empty pDG148 plasmid was used for background fluorescence. The calibration curve was determined by fitting the data of three independent biological replicates, each consisting of three technical replicates (see Fig. S1 of the Supplementary Data).

For  $\text{pH}_i$  measurements during stress experiments, cells containing empty pDG148 and pDG148-pHluorin were grown exponentially and ratiometric pHluorin expression was induced with 1 mM IPTG for at least 2.5 h as described above for the calibration of  $\text{pH}_i$ . At an  $\text{OD}_{600}$  of 0.35 cells were transferred into two microtiter plate readers, measuring the fluorescence and optical density (SpectraMax Plus, Molecular Devices Corp.). At an  $\text{OD}_{600}$  of 0.16 in the microtiter plate readers (which corresponds to an  $\text{OD}_{600}$  of 0.4 in a 1-cm-path-length spectrophotometer), the cells were stressed with the following chemicals: KS (0.5, 1, 3, or 10 mM), KAc (5, 25, or 80 mM), and CCCP (0.25, 0.5, 0.85, or 2  $\mu\text{M}$ ). Cells were cultivated in the microtiter plate readers under rigorous shaking at 37 °C for 120 min. All conditions were tested in the plate readers four times, and biologically independent experiments were performed at least in duplicate.

#### 2.4. Time-resolved transcriptome analysis using DNA microarrays

An exponentially growing culture of *B. subtilis* WT strain PB2 was split in two and cultured in well-controlled batch-fermentors (37 °C, pH 6.4, aeration rate: 0.5 L/min, stirring: 200 rpm) as described previously (Ter Beek et al., 2008). At an  $\text{OD}_{600}$  of 0.2, one culture was stressed with 25 mM KAc or 0.85  $\mu\text{M}$  CCCP. 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 KAc or CCCP. The cells were collected as described by Ter Beek et al. (2008). Total RNA was isolated as described previously (Keijser et al., 2007). Two biologically independent experiments were performed.

The synthesis of labeled cDNA, hybridization, scanning of the DNA microarrays, as well as the microarray data extraction and processing were carried out as described by Ter Beek et al. (2008). The data was normalized using J-Express Pro 2.7 software (MolMine AS). After the processing of the microarray data 3746 (91.4%) and 3685 (89.9%) genes of the total number of 4100 genes present on the array remained for each time-point, of which 503 (12.3%) and 582 (14.2%) were found to be significantly expressed in the KAc and CCCP treatments, respectively. The degree of enrichment or depletion for a specific gene group in the given significantly up- or downregulated genes was quantitatively assessed using a hypergeometric distribution analysis (Motulsky, 1995). Gene groups were considered to be enriched or depleted when the calculated  $P$  value was below 0.01.

The microarray data was analyzed using two complementary methods. Hierarchical clustering (Eisen et al., 1998) of specific subsets of significantly regulated genes was used to identify groups of genes with similar transcription profiles. This was performed in J-Express Pro 2.7 (MolMine AS) using the average linkage (WPGMA) clustering method and Euclidian distance metric. T-profiler (Boorsma et al., 2005) was used to assess the contribution of the expression of genes from specific gene classes to the total gene expression of all genes via <http://www.science.uva.nl/~boorsma/t-profiler-bacillusnew/> as described by Ter Beek et al. (2008).

#### 2.5. Microarray data accession number

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE55051 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55051>).

### 3. Results

#### 3.1. Comparison of the growth inhibitions caused by weak organic acids

In this study, we compared the effects of weak organic acids on food spoilage and model organism *B. subtilis*. Besides the commonly

used food preservatives sorbic- and acetic-acid (both having a  $\text{pK}_a$  of 4.76), we also investigated the effects of the powerful uncoupler CCCP ( $\text{pK}_a$  of 5.95). Comparing the octanol–water partition coefficient values (taken from “LOGKOW: A databank of evaluated octanol–water partition coefficients (LogP)”; <http://logkow.cisti.nrc.ca/logkow/>) shows that CCCP is the most lipophilic compound ( $\log K_{ow} = 3.38$ ), followed by sorbic acid ( $\log K_{ow} = 1.33$ ). Acetic acid is the least hydrophobic compound ( $\log K_{ow} = -0.17$ ). Consequently, the solubility into the membrane is expected to be the highest for CCCP and the lowest for acetic acid.

We have shown previously that the growth inhibition by sorbic acid can be mainly attributed to the undissociated form of sorbic acid (HS) (Ter Beek et al., 2008). The sorbate anion ( $\text{S}^-$ ) also contributes to the growth inhibition although to a much lesser extent. Using the same approach, we determined the pH dependence of the inhibitory effect of acetic acid and CCCP on exponentially growing *B. subtilis* cells in a defined medium. Increasing concentrations of either potassium acetate (KAc) in the mM range or CCCP in the  $\mu\text{M}$  range resulted in a decreasing growth rate when grown at a  $\text{pH}_{ex}$  of 6.4 (Fig. 1A and B). Similar qualitative trends of the growth curves were observed for all  $\text{pH}_{ex}$  values tested (5.9, 6.4, 7.4, and 7.8), although higher concentrations of compound were needed at higher  $\text{pH}_{ex}$  to obtain similar reductions in  $\text{OD}_{600}$  (our unpublished data). This supports the notion that the effect of weak organic acids is mainly caused by the undissociated form (e.g. Beales, 2004; Brul and Coote, 1999). The concentrations needed to cause a similar inhibition of growth negatively correlated with the  $\log K_{ow}$  values of each weak organic acid (e.g. 0.85  $\mu\text{M}$  CCCP, 3 mM KS, and 25 mM KAc were needed to inhibit the growth by  $\sim 30\%$  at a  $\text{pH}_{ex}$  of 6.4), indicating that their solubility into the membrane has a prominent effect on the growth inhibition or is the rate-limiting step. Estimated MIC values showed similar  $\text{pH}_{ex}$  and membrane solubility trends: higher estimated MIC values at higher  $\text{pH}_{ex}$  and lower  $\log K_{ow}$  values (see Table S1 of the Supplementary Data). Next, we plotted the percentages of growth inhibition (GI%) against the concentrations of the anion ( $\text{Ac}^-$  and  $\text{CCCP}^-$ ) and the undissociated (HAc and HCCCP) form of the acids (Fig. 1C–F). In contrast to the growth inhibition curves obtained for HS (Ter Beek et al., 2008), no overlap of the curves was observed for HAc and HCCCP. A full overlap implies that the inhibitory effect is determined by the amount of undissociated acid present and that this is pH independent.

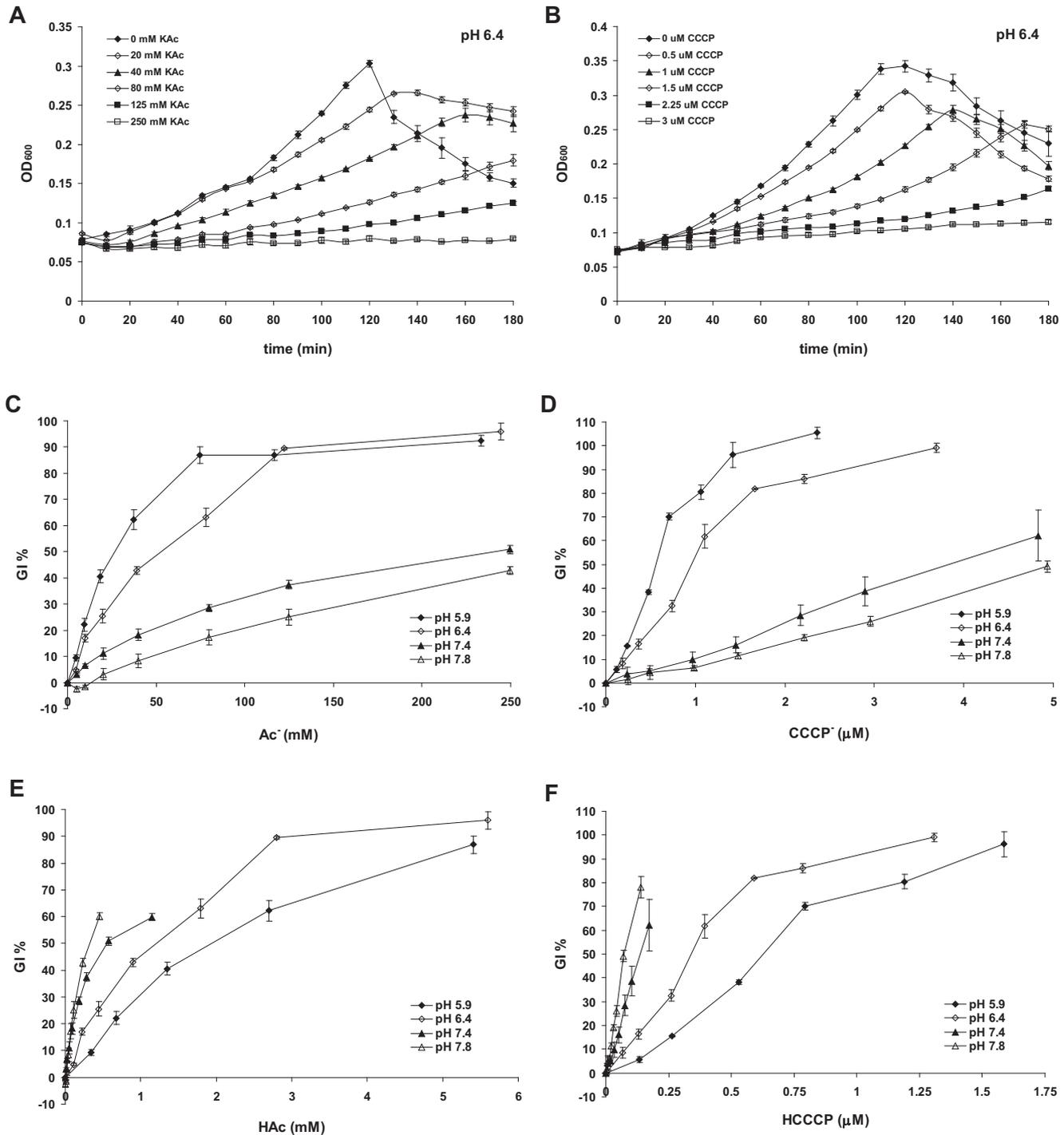
#### 3.2. Weak organic acid stress lowers the $\text{pH}_i$

To confirm lowering of the cytosolic pH by weak organic acid stress we set out to measure the  $\text{pH}_i$  in *B. subtilis* cells. Miesenböck et al. (1998) constructed a pH-sensitive version of GFP (ratiometric pHluorin), which allows *in situ* expression and direct *in vivo* pH measurements, without disturbing the cells. We previously showed using pHluorin that sorbic acid lowers both the cytosolic and mitochondrial pH in *Saccharomyces cerevisiae* (Orij et al., 2009). Here we cloned the gene coding for ratiometric pHluorin into the IPTG inducible expression vector pDG148 (Stragier et al., 1988) and transformed the resulting plasmid into WT *B. subtilis* cells. Fluorescent microscopy validated the expression of pHluorin in strain ATB032 (our unpublished results).

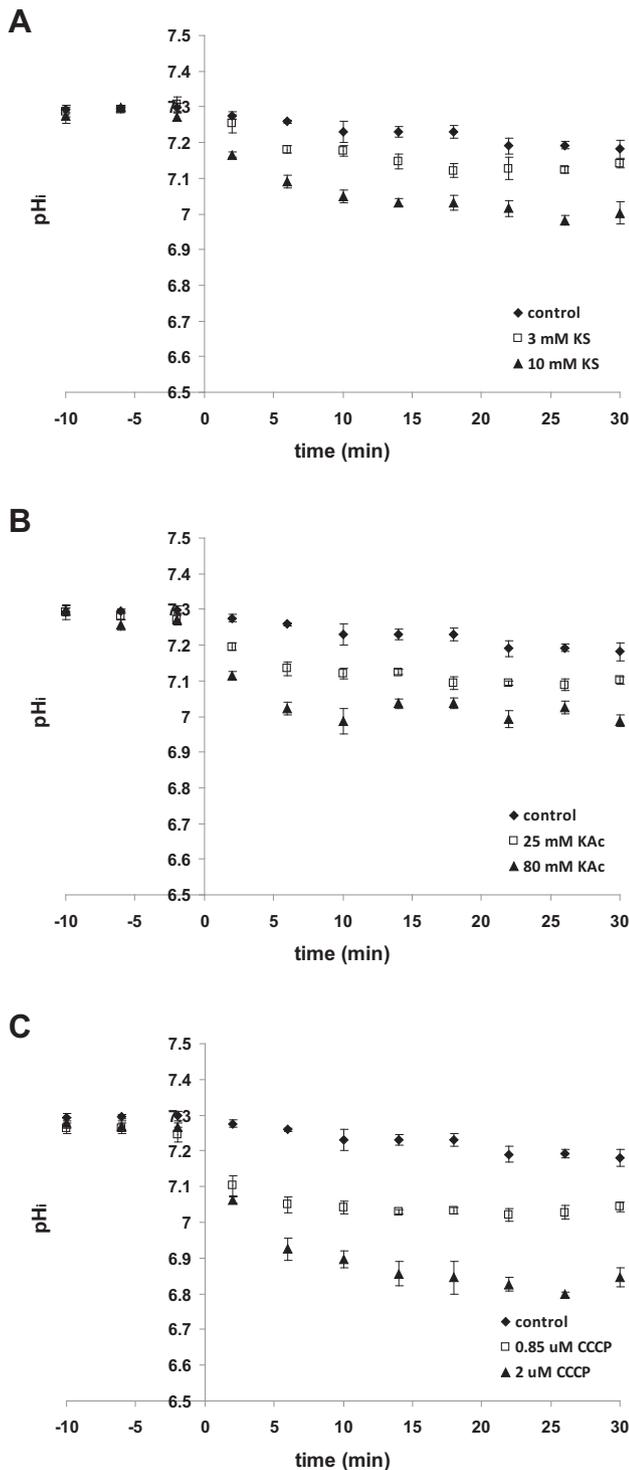
In unstressed cells we measured a  $\text{pH}_i$  of  $7.3 \pm 0.1$  when cultured in a defined minimal medium of pH 6.4, maintaining a  $\Delta\text{pH}$  of around 0.9 pH unit. This  $\text{pH}_i$  value lies in range of previously reported values for *B. subtilis* (Booth, 1985; Breeuwer et al., 1996; Kitko et al., 2009; Shioi et al., 1980; Slonczewski et al., 2009). All three tested compounds (KS, KAc, and CCCP) clearly lowered the  $\text{pH}_i$  in a concentration dependent manner (Fig. 2). Note that, compared to KS or KAc, very low concentrations of CCCP ( $\mu\text{M}$  range) clearly lowered the  $\text{pH}_i$  (Fig. 2C). The maximal  $\text{pH}_i$  drop seemed to

occur already within the first minutes of stress and did not recover to control levels. Longer incubations (2 h) also did not reveal a recovery from the drop in  $\text{pH}_i$  values (our unpublished results). The stresses applied did not lead to a loss in  $\text{OD}_{600}$  and the cells

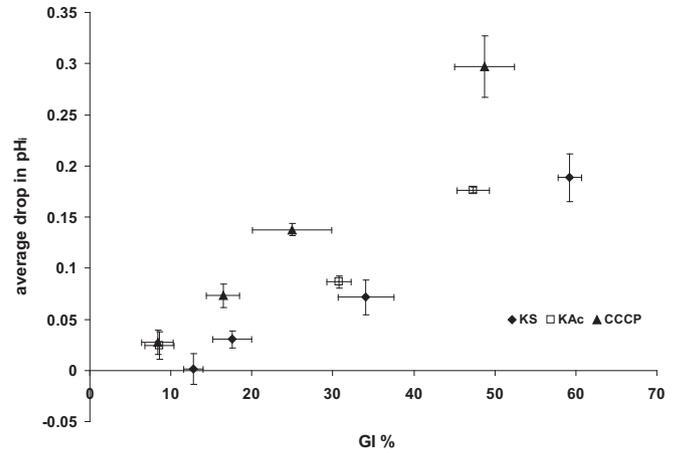
continued growing albeit at a reduced rate. Depending on the concentration of weak organic acid used the growth rate was reduced between 25 and 60% (Fig. 2). Addition of the tested compounds to the medium, as well as the growth of the control cells in



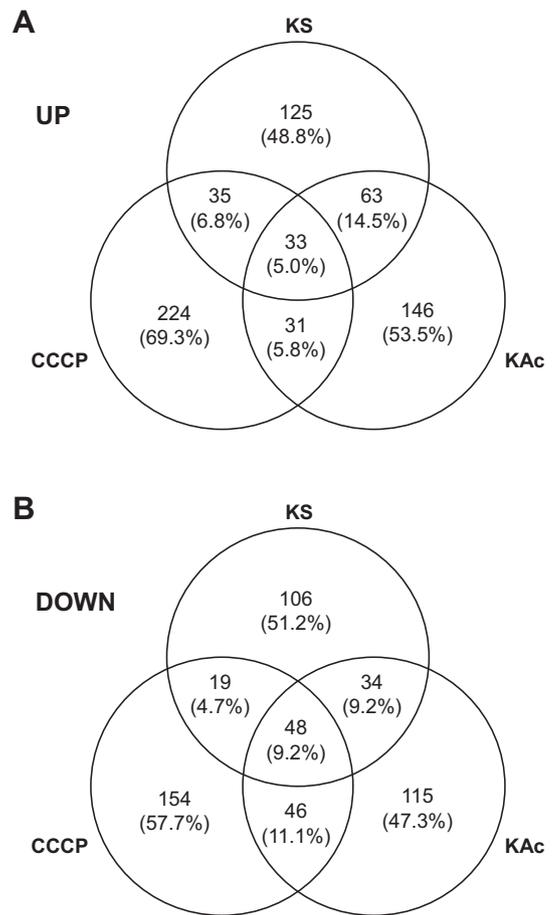
**Fig. 1.** Growth inhibition of exponentially growing *B. subtilis* by KAc and CCCP at various pH values. (A and B) The growth of PB2 in defined minimal medium at pH 6.4 was monitored in a microtiter plate reader. The closed diamonds indicate the growth of the control experiment (no addition of KAc or CCCP). (A) Stress conditions were 10 mM (open diamonds), 20 mM (closed triangles), 40 mM (open triangles), 80 mM (closed squares), 125 mM (open squares), and 250 mM (crosses) KAc. (B) Stress conditions were 0.25  $\mu\text{M}$  (open diamonds), 0.5  $\mu\text{M}$  (closed triangles), 1  $\mu\text{M}$  (open triangles), 1.5  $\mu\text{M}$  (closed squares), 2.25  $\mu\text{M}$  (open squares), and 3  $\mu\text{M}$  (crosses) CCCP. The  $\text{OD}_{600}$  was monitored during 180 min. The values represent the means of four measurements, including the standard errors. (C, D, E and F) Percentage of growth inhibition compared to the control (no addition of stress) as a function of the calculated concentration of  $\text{Ac}^-$  (C) or CCCP $^-$  (D) and HAc (E) or HCCCP (F) molecules. Experiments were performed at pH 5.9 (closed diamonds), pH 6.4 (open diamonds), pH 7.4 (closed triangles), and pH 7.8 (open triangles). The values represent the means of minimally two biologically independent experiments each consisting of four technical replicates, including the standard errors.



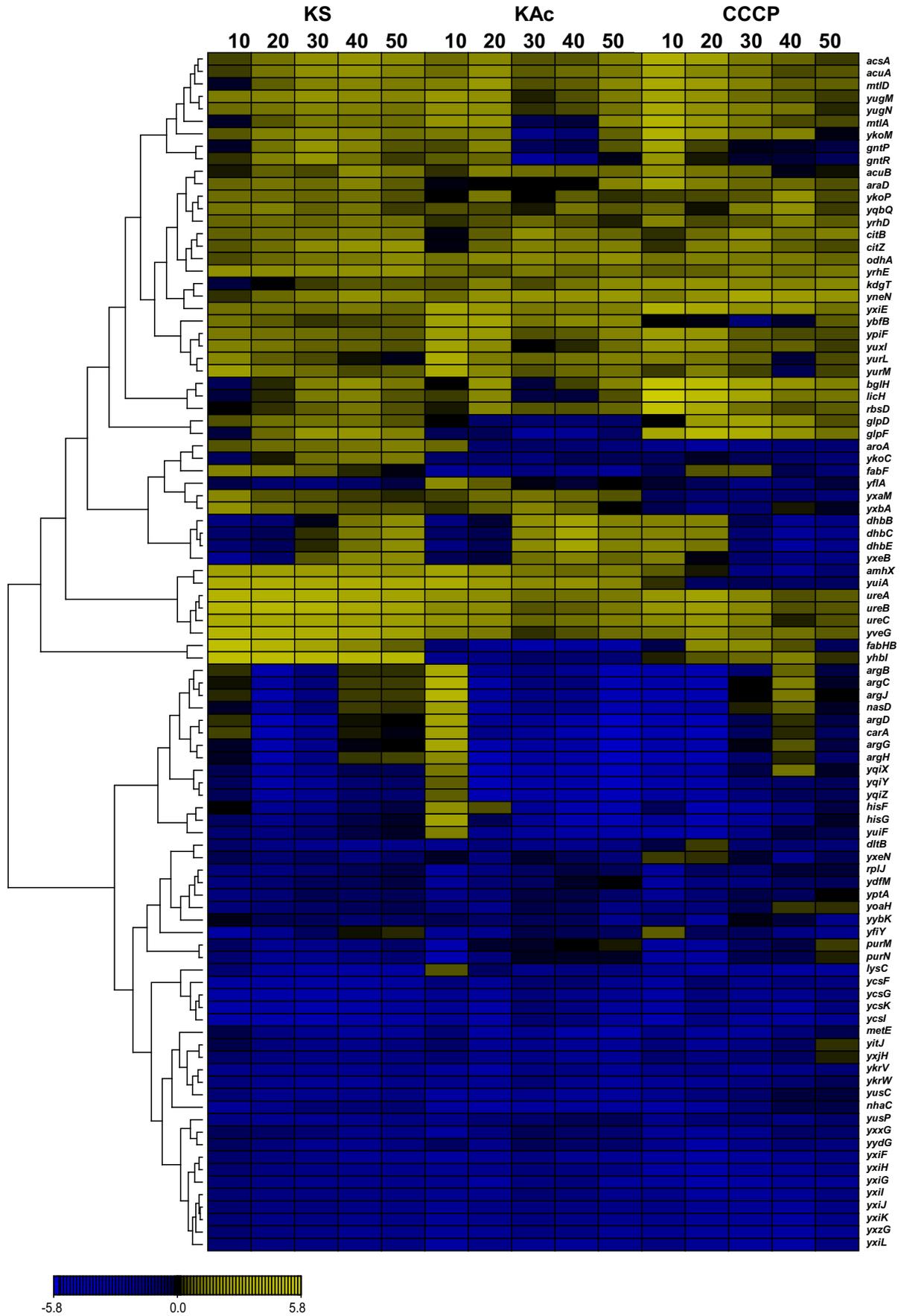
**Fig. 2.** Weak organic acids lower the intracellular pH. The  $pH_i$  profiles of non-stressed control cells ( $\mu_{max} = 0.52 \pm 0.02 \text{ h}^{-1}$ ) (closed diamonds) and cells challenged with a low (open squares) or high (closed triangles) concentration of added chemical when grown at a  $pH_{ex}$  of 6.4 are shown. Cells were stressed ( $t = 0$ ) with 3 or 10 mM KS, reducing the growth rate by 34 and 59%, respectively (A), 25 or 80 mM KAc (B), reducing the growth rate by 31 and 47%, respectively, and 0.85 or 2  $\mu\text{M}$  CCCP, reducing the growth rate by 25 and 49%, respectively (C). Values represent the average of four technical replicates and error bars indicate the standard error.



**Fig. 3.** The comparison of the drop in  $pH_i$  and GI % values reveals differences between weak organic acids. The average drop in  $pH_i$  was calculated between 2 and 30 min after stress and plotted against the GI % caused by 0.5, 1, 3, or 10 mM KS (closed diamonds), 5, 25, or 80 mM KAc (open squares), and 0.25, 0.5, 0.85, or 2  $\mu\text{M}$  CCCP (closed triangles) when cells were grown a  $pH_{ex}$  of 6.4. Values represent the average of minimally two biological independent experiments, each based on minimally two technical replicates. The error bars indicate the standard deviation.



**Fig. 4.** Venn diagrams showing the number of significantly regulated genes for each type and combination of weak organic acid stress. Shown are significantly upregulated (A) or downregulated (B) genes in at least one time-point during 50 min of stress. The percentages of the number of uniquely regulated genes are shown in brackets. For clarity: the 125 genes uniquely upregulated by KS is 48.8% of all genes upregulated by KS ( $125 + 63 + 33 + 35 = 256$  genes). The 35 genes uniquely upregulated by only KS and CCCP is 6.8% of all genes upregulated by KS and CCCP ( $125 + 63 + 33 + 35 + 31 + 224 = 511$  genes), etc.



itself did not affect the  $pH_{ex}$  of 6.4 during the time course of the experiment (our unpublished data).

We plotted the GI % caused by each weak organic acid against the average drop in  $pH_i$  measured between 2 and 30 min after stress (Fig. 3). Taking the GI % into account makes it easier to compare the effects of the three tested compounds. From Fig. 3 it can be observed that CCCP gives the highest average drop in  $pH_i$  at similar growth inhibition percentages. Although the addition of 2  $\mu$ M of CCCP reduced the growth rate with ~50% and caused an average  $pH_i$  drop of 0.3 pH unit, the cells still sustained a  $\Delta pH$  of ~0.6 ( $pH_i$  minus  $pH_{ex}$ ). Only ceasing growth almost completely by the addition of 200 mM KAc or 4  $\mu$ M CCCP resulted in an inverse  $\Delta pH$  (our unpublished data). Noteworthy is the observation that the addition of 0.5 mM KS at a  $pH_{ex}$  of 6.4 led to a significant inhibition of the growth rate by 13%, however no reduction in the cytosolic pH was measured (Fig. 3). On the other hand, low concentrations of CCCP or KAc stress did result in a significant drop in  $pH_i$ , even at lower GI %. These observations suggest that the growth inhibitory effect of sorbic acid is not solely the acidification of the cytosol.

### 3.3. Overall transcriptional responses of *B. subtilis* sub-lethally stressed with weak organic acids

We analyzed the transcriptional responses of *B. subtilis* sub-lethally stressed with weak organic acids. *B. subtilis* cells were grown to early exponential-phase in a buffered medium of pH 6.4 in well-controlled batch fermentors and treated with 25 mM KAc or 0.85  $\mu$ M CCCP, resulting in a growth reduction of 33% and 28%, respectively. Samples were taken at 10, 20, 30, 40, and 50 min after exposure to stress and compared to an untreated control. Since the experimental set-up of the previously published transcriptional response of *B. subtilis* upon 3 mM KS stress was similar and the growth rate was reduced by 29% (Ter Beek et al., 2008), we could directly compare the obtained data with each other. Since the tested compounds have different chemical properties, the weak organic acids may induce responses in *B. subtilis* within different time-frames. Therefore, we analyzed the transcriptome in a time-dependent manner.

The treatment of *B. subtilis* cells with KAc or CCCP resulted in the significantly different expression of 503 and 582 genes, respectively. Including the 459 differentially expressed genes in the KS treatment (Ter Beek et al., 2008), in total 1107 genes were differentially expressed in at least one time point by at least one stress. Of these 1107 regulated genes, 657 were induced and 522 repressed upon weak organic acid stress (Fig. 4) and consequently, 72 genes illustrated up- and downregulation during the time-course analyzed. The largest overlap of induced genes was seen in KS and KAc stressed cultures (14.5%). However, the downregulated genes showed the largest overlap (11.1%) between the CCCP- and KAc-treated cells (Fig. 4). The numbers of up- and downregulated genes for each time-point, specific for each stress or combination of stress, are given in the Supplementary Data (Table S2). The significantly regulated genes were divided into 7 categories and analyzed with hierarchical clustering (Eisen et al., 1998). These categories consisted of genes uniquely regulated by (a) KS, (b) KAc, (c) CCCP, (d) KS and KAc, (e) KAc and CCCP, (f) CCCP and KS, or by (g) KS, KAc and CCCP (Fig. 5, and Fig. S2–S7 of the Supplementary Data). All expression data were analyzed and compared using T-profiler, which transforms the transcriptional data of single genes into the

behavior of gene groups that reflect biological processes in the cells (Boorsma et al., 2005). All gene groups with significant *T* values ( $E < 0.05$ ) in any time point are presented in the Supplementary Data (Tables S3–S6).

### 3.4. Identification of a minimal generic transcriptional response to weak organic acids

Ninety-six out of the 1107 differentially expressed genes were uniquely induced or repressed by all three acids (Fig. 5). Out of these 96 genes 33 (5.0% of all 657 induced genes) were upregulated and 48 (9.2% of all 522 repressed genes) were downregulated by all three acids (Fig. 4). Accordingly, 15 genes showed opposite expression within the expression patterns of the three weak organic acids.

Major changes in genes involved in carbon metabolism were observed (Fig. 5). Many genes negatively regulated by carbon catabolite control protein CcpA were found significantly upregulated by weak organic acid stress. Among them were *acsA*, encoding acetyl-CoA synthetase responsible for the degradation of acetate (Grundy et al., 1993), and the *acuABC* genes, involved in the activation of *AcsA* (Gardner et al., 2006). T-profiler analysis also revealed the significant derepression of CcpA-regulated genes, which normally occurs when the preferred carbon source (glucose, fructose, and mannose) is depleted (Deutscher et al., 2002), and induction of TCA cycle genes (Table 1). HPLC analysis showed that glucose was not depleted during the time-course of the experiment (our unpublished results).

Notable is the induction of the *ureABC* operon, which codes for urease (Cruz-Ramos et al., 1997) (Fig. 5). Ureasases are known to facilitate survival in acidic environments in some bacterial pathogens, through the production of basic ammonia (Collins and D'Orazio, 1993; Mobley et al., 1995). However, a urease mutant (*ureC*) did not show an altered phenotype upon KS (Ter Beek et al., 2008), KAc or CCCP stress when compared to the WT (our unpublished results).

Most of the arginine biosynthesis and transport genes *argCJBD-carAB-argF* and *argGH* were significantly downregulated by all three stresses (Fig. 5). In addition, the majority of genes belonging to the KipR regulon *ycaFGL-kiplAR-ycaK* (Wang et al., 1997), involved in the inhibition of the activation of sporulation, were found to be repressed by weak organic acid stress (Fig. 5). Geometric distribution analysis also showed that KS, KAc, and CCCP significantly ( $P < 0.0001$ ) repressed the genes controlled by AhrC, the arginine metabolism regulator (North et al., 1989), and the KipR regulon.

The largest part of the unknown genes *yxxG-yxiF-yxzC-yxiGH-yxzG-yxiJKLM* that lay adjacently to each other in the genome was significantly downregulated by all three acids (Fig. 5). Benzoic acid and salicylic acid, two weak organic acids with a  $pK_a$  of 4.21 and 3, respectively, and an adapted culture of pH 6 compared to pH 7 repressed most of these unknown genes (Duy et al., 2007; Kitko et al., 2009; Wilks et al., 2009). The function of these genes in response to (weak) acid stress remains to be elucidated.

### 3.5. Transcriptional responses indicative of intracellular acidification

Using pHluorin we demonstrated that the weak organic acids tested all three lowered the  $pH_i$  (Fig. 2). When comparing similar GI

**Fig. 5.** Hierarchical clustering of genes significantly and uniquely regulated by all three weak organic acids. All 96 genes showing significant expression during KS, KAc and CCCP treatment were hierarchically clustered using the average linkage (weighted-pair group method using average linkages) clustering method and a Euclidian distance metric (J-Express PRO 2.7; MolMine AS). Times refer to minutes after the addition of stress to exponentially growing cells.  $\log_2$  ratios are displayed colorimetrically, ranging from -5.8 to +5.8. Yellow indicates higher transcript levels in stressed cells than in control cells; blue indicates a reduction in mRNA content. The gene names are depicted next to the cluster. The  $\log_2$  ratios of each gene used are the averages of two biological independent experiments.

**Table 1**  
Gene groups significantly induced or repressed by all three weak acids, as identified by T-profiler.

Gene group <sup>a</sup>	Stress (no. of ORFs) <sup>b</sup>	T-value at time (min) <sup>c</sup>				
		10	20	30	40	50
<i>Transcription factor regulated gene group</i>						
AhrC_Negative	KS (7)	1.04	-12.03	-7.06	0.53	0.21
	KAc (7)	<b>6.81</b>	<b>-5.32</b>	<b>-6.91</b>	<b>-9.36</b>	<b>-14.61</b>
	CCCP (NA)	NA	NA	NA	NA	NA
CcpA_Negative	KS (108)	0.16	3.68	<b>9.13</b>	<b>10.28</b>	<b>8.25</b>
	KAc (110)	2.02	<b>6.54</b>	-0.36	0.96	4.05
	CCCP (112)	<b>17.86</b>	<b>12.64</b>	<b>10.02</b>	<b>5.56</b>	<b>7.17</b>
KipR_Negative	KS (7)	<b>-10.16</b>	<b>-10.09</b>	<b>-11.08</b>	<b>-10.88</b>	<b>-10.14</b>
	KAc (7)	-4.02	<b>-7.28</b>	<b>-4.32</b>	<b>-4.89</b>	<b>-6.30</b>
	CCCP (NA)	NA	NA	NA	NA	NA
PurR_Negative	KS (31)	<b>-8.22</b>	<b>-9.04</b>	<b>-7.75</b>	<b>-4.69</b>	<b>-3.54</b>
	KAc (32)	<b>-11.52</b>	2.10	1.97	2.85	3.01
	CCCP (32)	<b>-9.93</b>	<b>-8.91</b>	-0.94	1.57	2.95
RelA_Negative	KS (86)	<b>-5.30</b>	<b>-6.09</b>	<b>-5.27</b>	<b>-5.19</b>	<b>-5.00</b>
	KAc (88)	<b>-16.66</b>	<b>-10.82</b>	<b>-3.19</b>	<b>-4.75</b>	<b>-7.75</b>
	CCCP (88)	<b>-12.58</b>	<b>-8.23</b>	<b>-4.98</b>	<b>-3.49</b>	<b>-3.95</b>
<i>KEGG Pathway</i>						
Carbohydrate Metabolism	KS (230)	0.14	2.36	<b>5.81</b>	<b>5.79</b>	<b>5.82</b>
	KAc (237)	3.69	<b>5.24</b>	<b>2.77</b>	1.90	2.10
	CCCP (230)	<b>12.53</b>	<b>10.75</b>	<b>10.88</b>	<b>4.60</b>	<b>7.53</b>
Citrate cycle (TCA cycle)	KS (18)	2.47	3.55	<b>5.21</b>	<b>5.9</b>	<b>6.60</b>
	KAc (20)	1.81	3.57	<b>5.43</b>	<b>4.58</b>	3.38
	CCCP (19)	2.17	2.70	<b>4.27</b>	1.86	1.65
Environmental Information Processing	KS (437)	<b>-5.15</b>	<b>-4.96</b>	-2.19	-0.68	0.46
	KAc (438)	<b>-4.40</b>	-1.64	-3.81	-1.26	-0.70
	CCCP (432)	<b>-0.71</b>	<b>-1.33</b>	<b>-5.52</b>	<b>-9.65</b>	<b>-5.92</b>
Membrane Transport	KS (333)	<b>-5.98</b>	<b>-5.81</b>	<b>-2.82</b>	-0.94	0.20
	KAc (336)	<b>-4.95</b>	-1.34	<b>-5.26</b>	-1.27	-0.33
	CCCP (334)	<b>-0.31</b>	<b>-0.97</b>	<b>-6.74</b>	<b>-10.52</b>	<b>-7.33</b>
Nucleotide Metabolism	KS (101)	<b>-5.00</b>	<b>-6.09</b>	<b>-4.56</b>	-2.81	-1.68
	KAc (100)	<b>-6.94</b>	-1.07	1.11	-0.08	-1.07
	CCCP (99)	<b>-6.11</b>	<b>-5.93</b>	-0.45	-0.58	0.62
Purine metabolism	KS (71)	-2.66	<b>-4.21</b>	-3.02	-2.41	-1.01
	KAc (70)	<b>-5.08</b>	-0.56	1.07	0.20	-0.39
	CCCP (70)	<b>-4.8</b>	<b>-4.99</b>	-0.45	-0.76	1.71
Translation	KS (111)	-3.93	<b>-4.40</b>	<b>-4.39</b>	<b>-4.22</b>	<b>-4.12</b>
	KAc (120)	<b>-12.12</b>	<b>-9.28</b>	-2.24	<b>-4.51</b>	<b>-7.62</b>
	CCCP (117)	<b>-10.62</b>	<b>-8.10</b>	<b>-5.27</b>	<b>-4.61</b>	<b>-3.86</b>
Urea cycle and metabolism of amino groups	KS (20)	2.49	<b>-5.44</b>	-2.32	2.09	2.04
	KAc (19)	<b>5.89</b>	-3.26	-3.92	<b>-5.31</b>	<b>-9.05</b>
	CCCP (18)	-3.83	<b>-4.93</b>	0.01	0.53	-0.43
<i>SubtiList functional category</i>						
1 Cell envelope and cellular processes	KS (872)	<b>-6.76</b>	<b>-5.9</b>	<b>-4.09</b>	-2.74	-1.89
	KAc (833)	<b>-6.08</b>	<b>-4.97</b>	<b>-5.25</b>	-3.5	-2.71
	CCCP (819)	-1.59	-1.04	<b>-4.2</b>	<b>-6.52</b>	-4.03
1.2 Transport/binding proteins and lipoproteins	KS (370)	<b>-5.19</b>	<b>-5.44</b>	-2.73	-0.88	0.33
	KAc (372)	<b>-4.83</b>	<b>-2.61</b>	<b>-7.19</b>	-2.59	-1.37
	CCCP (372)	-0.16	-1.74	<b>-7.77</b>	<b>-12.23</b>	<b>-7.54</b>
2.1 Metabolism of carbohydrates and related molecules	KS (256)	0.92	3.11	<b>5.80</b>	<b>5.26</b>	<b>4.61</b>
	KAc (260)	<b>6.00</b>	<b>6.56</b>	3.59	3.00	3.39
	CCCP (256)	<b>12.43</b>	<b>10.07</b>	<b>9.57</b>	<b>3.62</b>	<b>6.48</b>
2.3 Metabolism of nucleotides and nucleic acids	KS (89)	<b>-7.79</b>	<b>-7.27</b>	<b>-6.20</b>	<b>-4.67</b>	<b>-3.73</b>
	KAc (91)	<b>-8.10</b>	-0.77	0.66	-0.13	0.01
	CCCP (91)	<b>-6.82</b>	<b>-6.76</b>	-1.12	0.78	0.74
3.7 Protein synthesis	KS (89)	-3.73	<b>-4.36</b>	<b>-4.23</b>	<b>-3.98</b>	<b>-3.87</b>
	KAc (98)	<b>-12.45</b>	<b>-9.70</b>	-2.32	<b>-4.62</b>	<b>-7.19</b>
	CCCP (94)	<b>-10.92</b>	<b>-8.07</b>	<b>-4.85</b>	<b>-3.93</b>	<b>-3.07</b>

<sup>a</sup> Transcription factor regulated gene groups are taken from the database of transcriptional regulation in *Bacillus subtilis* (DBTBS) (<http://dbtbs.hgc.jp/>) and for FapR from (SCHUJMAN et al., 2003) and for the stringent response (RelA-dependent) from (EYMAN et al., 2002). A distinction is made between groups of genes that are positively or negatively regulated by the mentioned transcription factor (indicated by Positive or Negative). Pathways are taken from the Kyoto Encyclopedia of Genes and Genomes database (KEGG) (<http://www.genome.jp/>). Functional categories are taken from the SubtiList database (<http://genolist.pasteur.fr/SubtiList/>).

<sup>b</sup> Number of ORFs within each group present in the microarray experiments is shown in parentheses. NA: not available.

<sup>c</sup> The T-values presented are calculated on the basis of two biologically independent experiments. Shown are the gene groups that have at least one significant T-value ( $E < 0.05$ ) for all three stresses in the analyzed time-course. Significant T-values are shown in bold and red (positive) or in bold and green (negative). NA: not available. T-profiler requires at least 7 genes in a group in order to calculate reliable T values ( $E < 0.05$ ) (BOORSMA et al., 2005). However, geometric distribution analysis showed that KS, KAc, and CCCP significantly ( $P < 0.0001$ ) repressed the genes controlled by AhrC and KipR.

the significant induction of the SigB-mediated GSR by KAc and CCCP only (Table 2). The SigB regulon is induced by many different types of stress (e.g. glucose starvation, heat, low external pH, salt, and ethanol) and provides the cell with non-specific, multiple, and preventive stress resistance (Hecker et al., 2007). Polen et al. (2003) also reported the induction of the RpoS-mediated GSR in *E. coli* upon acetate, propionate and CCCP stress. In addition, some SigB-regulated genes were also induced in benzoate-adapted *B. subtilis* cells (Kitko et al., 2009).

The SigM regulon in *B. subtilis* is involved in maintaining membrane and cell wall integrity and is induced by heat, ethanol, cell wall antibiotics, superoxide stress and low pH (Thackray and Moir, 2003). CCCP clearly induced genes regulated by SigM (Table 2). However, KS and KAc both did not induce this regulon. The induction of SigM by CCCP might be the result of acidification of the cytosol and/or cell envelope stress (further discussed below). Since the strongest intracellular acidification was observed in CCCP-stressed cells (Fig. 3), this observation might explain the

**Table 2**  
Gene groups significantly induced or repressed by weak organic acids indicative of intracellular acidification and/or cell envelope stress, as identified by T-profiler.

Gene group <sup>a</sup>	Stress (no. of ORFs) <sup>b</sup>	T-value at time (min) <sup>c</sup>				
		10	20	30	40	50
<i>Transcription factor regulated gene group</i>						
BkdR_Positive (E)	KS (7)	<b>5.80</b>	<b>5.76</b>	<b>5.97</b>	<b>5.66</b>	<b>5.99</b>
	KAc (7)	2.49	3.20	3.69	2.94	2.28
	CCCP (7)	-2.91	-1.59	1.16	-0.37	1.05
FapR_Negative (E)	KS (10)	<b>6.98</b>	<b>5.63</b>	4.05	2.12	0.91
	KAc (11)	<b>-5.09</b>	<b>-6.25</b>	<b>-6.21</b>	<b>-6.81</b>	<b>-6.16</b>
	CCCP (8)	-2.11	1.66	2.23	0.35	-2.33
YvrH_Positive (E)	KS (17)	-3.43	<b>-4.51</b>	<b>-5.59</b>	<b>-6.55</b>	<b>-6.53</b>
	KAc (15)	-3.11	-3.54	-2.66	-2.90	-2.29
	CCCP (12)	-2.43	-2.02	-2.96	-0.56	-3.84
<i>Sigma factor dependent gene group</i>						
SigB (A)	KS (95)	0.36	-1.70	-3.90	-3.27	-2.71
	KAc (93)	<b>10.59</b>	<b>5.81</b>	0.08	-1.84	-0.02
	CCCP (96)	<b>5.22</b>	2.81	0.03	2.05	2.89
SigM (A & E)	KS (14)	-1.19	-2.57	-3.46	-3.18	-3.32
	KAc (15)	-0.09	-1.69	-1.53	-1.33	-0.89
	CCCP (15)	<b>7.40</b>	<b>7.51</b>	<b>5.82</b>	3.00	-2.23
SigW (E)	KS (60)	-1.33	<b>-4.84</b>	<b>-7.12</b>	<b>-7.66</b>	<b>-7.05</b>
	KAc (59)	2.26	-0.53	0.59	-0.6	-1.72
	CCCP (60)	-0.37	-0.04	-3.82	-2.08	<b>-5.92</b>
SigX (E)	KS (20)	-2.38	-3.93	<b>-5.4</b>	<b>-5.77</b>	<b>-6.09</b>
	KAc (20)	-1.74	-3.30	<b>-4.71</b>	<b>-4.14</b>	-2.84
	CCCP (18)	0.57	0.67	-1.05	-0.02	-3.92
<i>KEGG Pathway</i>						
Fatty acid biosynthesis (E)	KS (21)	3.97	3.44	2.81	1.09	0.34
	KAc (20)	-3.90	<b>-4.44</b>	<b>-4.07</b>	<b>-5.28</b>	<b>-4.62</b>
	CCCP (18)	-0.86	1.15	1.90	0.64	-2.3
<i>SubtiList functional category</i>						
1.1 Cell wall (E)	KS (110)	-3.53	-3.37	-3.84	-3.71	-3.48
	KAc (102)	<b>-5.19</b>	<b>-3.36</b>	-0.72	-1.90	-1.83
	CCCP (98)	<b>-3.31</b>	<b>-1.02</b>	-0.06	0.67	-0.11
2.4 Metabolism of lipids (E)	KS (85)	<b>4.5</b>	<b>4.26</b>	<b>4.50</b>	3.5	3.49
	KAc (87)	-1.82	-1.01	-1.38	-1.52	-1.35
	CCCP (82)	-0.28	1.9	1.82	0.09	-1.20

<sup>a</sup> Transcription factor regulated gene groups are taken from the database of transcriptional regulation

in *Bacillus subtilis* (DBTBS) (<http://dbtbs.hgc.jp/>) and for FapR from (SCHUJMAN et al., 2003) and for the stringent response (RelA-dependent) from (EYMAN et al., 2002). A distinction is made between groups of genes that are positively or negatively regulated by the mentioned transcription factor (indicated by Positive or Negative). Pathways are taken from the Kyoto Encyclopedia of Genes and Genomes database (KEGG) (<http://www.genome.jp/>). Functional categories are taken from the SubtiList database (<http://genolist.pasteur.fr/SubtiList/>). Regulated gene groups indicative of intracellular acidification and/or cell envelope stress are indicated by 'A' and 'E' in parentheses, respectively.

<sup>b</sup> Number of ORFs within each group present in the microarray experiments is shown in parentheses.

<sup>c</sup> The T-values presented are calculated on the basis of two biologically independent experiments. Shown are the gene groups that have at least one significant T-value ( $E < 0.05$ ) for all three stresses in the analyzed time-course. Significant T-values are shown in bold and red (positive) or in bold and green (negative).

%, CCCP caused the largest drop in pH<sub>i</sub> and KS the smallest (Fig. 3). Except for the genes encoding urease, no apparent similar responses known to be involved in the maintenance of pH homeostasis were identified (Fig. 5). However, T-profiler analysis showed

absence of the induction of the SigM regulon in KS- or KAc-treated cultures.

### 3.6. Weak organic acids induce (distinct & opposite) responses that all indicate changes in the cell envelope

Sorbic acid caused a clear and unique induction of the fatty acid biosynthesis genes regulated by the fatty acid and phospholipid regulator FapR (Table 2). The gene group regulated by BkdR, involved in the synthesis of precursor molecules for branched-chain fatty acids (Debarbouille et al., 1999), was also uniquely induced by KS stress. The induction of both the FapR and BkdR-regulated genes may increase the number of long-chain and branched-chain fatty acids in the membrane (de Mendoza et al., 2002; Schujman et al., 2003). On the other hand, KAc caused a significant repression of the fatty acid biosynthesis genes, which could in theory result in shorter acyl-lipids in the membrane (Table 2).

We discovered altered expression of gene groups involved in the adaptation of the cell envelope (Table 2). CCCP clearly induced genes regulated by SigM (Table 2), involved in maintaining membrane and cell wall integrity (Thackray and Moir, 2003). However, KS and KAc both did not induce this regulon. The extracytoplasmic function sigma factor SigX and SigW are also involved in the regulation of cell envelope homeostasis (Helmann, 2002). A downregulation of SigW-mediated genes was seen in KS and CCCP-stressed cells and not in an acetate-treated culture (Table 2). A SigX-mediated repression of genes was clearly observed in KS and KAc-treated cells, but not in a CCCP-stressed culture during the time-course analyzed. YvrH is another regulator involved in the homeostasis of the cell surface (Serizawa et al., 2005). Although there is overlap between the SigX regulon and that of YvrH, only KS repressed this regulon significantly (Table 2).

## 4. Discussion

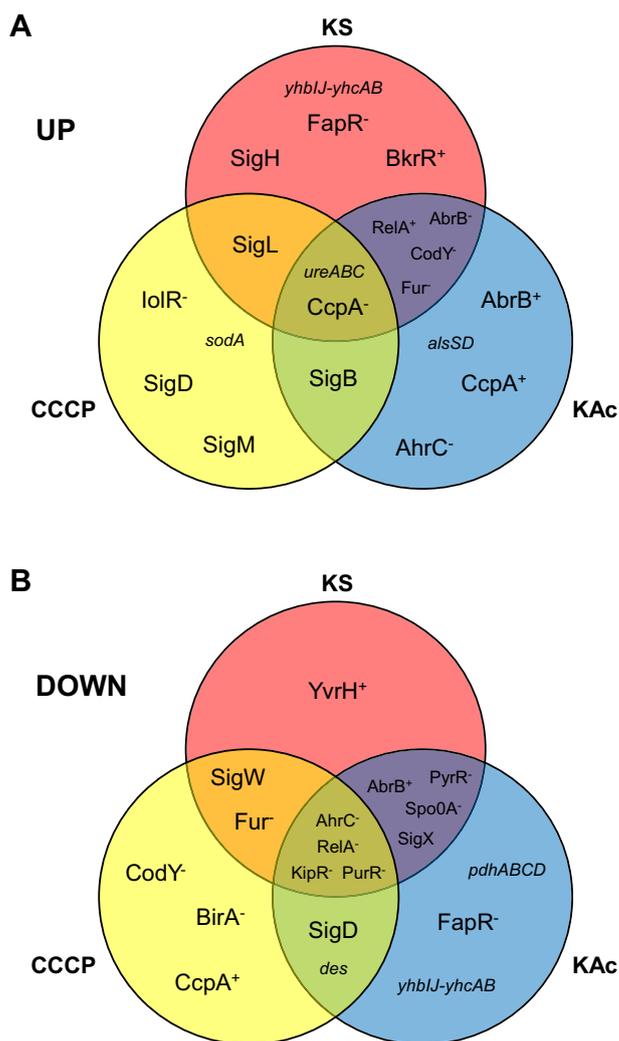
In this study we compared the effects of different weak organic acids, a class of established preservatives in the food industry. To get to a better understanding of the modes of action of these preservatives and the elucidation of resistance mechanisms in the spoilage organism *B. subtilis* we performed a comparative physiological and transcriptional analysis. We linked the direct measurement of  $pH_i$  with growth inhibition and transcriptional responses. As weak organic acids we tested commonly utilized sorbic- and acetic acid, and compared their effects to that of CCCP, a classical uncoupler and weak organic acid. The negative charge of the CCCP anion is delocalized over the entire molecule and therefore, it may be assumed that the anion (besides the neutral molecule) is able to diffuse into the membrane more easily than e.g.  $S^-$ . By comparison with CCCP we tried to dissect the possible uncoupling effects from other specific effects caused by sorbic and acetic acid.

The growth inhibition curves determined at different pH values revealed that the undissociated form of the acids dominated the inhibitory effect (Fig. 1), with the HS contribution to the growth inhibition being the strongest out of the three acids used (i.e. no overlap of the curves for HAC and HCCCP in Figs. 1E and F). Additionally, the anions also contributed to the reduction in growth. Evidently, the relative contribution of  $CCCP^-$  and  $Ac^-$  (Figs. 1C and D) is far more than that of  $S^-$  (Ter Beek et al., 2008). The antimicrobial efficacy of the acids correlated to their solubility in the membrane.

To reveal the generic and specific responses of weak organic acids, microarray technology is a powerful tool. We analyzed the transcriptional responses of *B. subtilis* upon mild weak organic acid

stress. An overview of the transcriptional responses of *B. subtilis* stressed with weak organic acids is depicted in Fig. 6. The three tested compounds KS, KAc, and CCCP all induced responses normally observed upon energy/nutrient limitation, such as the relief of the carbon catabolite repression, the induction of the RelA-dependent stringent response, and the significant downregulation of gene groups involved in nucleotide metabolism, protein synthesis and translation (Table 1). These responses indicate that the cell may experience an actual nutrient limitation. However, nutrients (glucose) in the medium were in excess. Weak organic acid stress may lead to a hampering of the uptake of nutrients, likely caused by the decrease in the proton gradient through influx of protons. In addition, weak organic acids may interfere directly with the nutrient uptake machinery or enzymes further downstream the metabolic pathway.

Repression of the KipR regulon (involved in the inhibition of the activation of sporulation) by weak organic acids may indicate the release of the “brake” on the sporulation-regulatory cascade. The transcriptome of KS-, KAc-, or CCCP-treated cells did not reveal an induction of sporulation and no increase in spore counts were detected in KS-stressed cells (Ter Beek et al., 2008). On the contrary,



**Fig. 6.** Venn-diagram showing the transcriptional responses seen in weak organic acid-stressed cultures. Indicated are the responses of significantly (A) induced or (B) repressed individual genes and gene groups regulated by the depicted transcription- and sigma-factor. The plus and minus symbol indicates whether the gene group is regulated positively or negatively by the depicted regulator. See the text for details.

our previous data show that KS stress during exponential growth delayed sporulation induced in the stationary phase by glucose exhaustion (Hornstra et al., 2009). In our previous study, inactivation of *ycsF*, the first gene of the KipR regulon, did not lead to altered susceptibility for KS stress (Ter Beek et al., 2008). In addition, the *ycsF* mutant did not show an altered phenotype upon KAc or CCCP stress when compared to the WT (our unpublished data).

The main mode of action of weak organic acids is considered to be the acidification of the cytosol (Beales, 2004; Brul and Cooté, 1999; Davidson, 2001; Ter Beek and Brul, 2010). Therefore, we expressed ratiometric pHluorin in *B. subtilis* cells to measure the  $pH_i$ . Using this approach, *in situ* expression and direct *in vivo* pH measurements are possible, without disturbing the cells. Recently, similar strategies were used by Martínez II et al. (2012), who expressed pHluorin in *E. coli* and *B. subtilis* to monitor the  $pH_i$  upon low pH and benzoic acid stress in individual cells, and by us to measure the  $pH_i$  in germinating and outgrowing *B. subtilis* spores (van Beilen and Brul, 2013). Fluorescent measurements with cells expressing pHluorin showed that all three weak organic acids lower the cytosolic pH in *B. subtilis* (Fig. 2). Recovery of  $pH_i$  after the addition of weak organic acid stress was however not observed even after longer (2 h) incubations and seemed to coincide with the (continuous and stable) reduced growth rate (Fig. 1). Transcriptional indications of an acidification of the cytosol were observed in cultures stressed with CCCP or KAc. Both induced the GSR and only CCCP caused the activation of the SigM regulon (Table 2). When taking the GI % into account, CCCP clearly reduced the  $pH_i$  the most effective of the three stresses, followed by KAc and then KS (Fig. 3). These findings support the distinct observed inductions of the GSR and SigM regulon. It might be possible that a threshold level of acidification inside the cell is necessary before the GSR or SigM regulon are induced, or that other (additional) stress input signals are needed. It was shown that the induction of the GSR by nutritional stress is preceded by a drop in ATP levels (Zhang and Haldenwang, 2005). This indicates that the observed induction of the GSR in CCCP- and KAc-treated cells may be the consequence of a (severe) drop in ATP and/or the  $pH_i$ . On the contrary, mild KS stress did not induce the SigB-mediated GSR in *B. subtilis* (Ter Beek et al., 2008). The absence of the GSR in (mild) sorbic acid-stressed cells might indicate that ATP or  $pH_i$  levels were not severely affected.

Acetate-stressed cells clearly seem to shift their metabolism to the utilization of acetate by the upregulation of *alsSD* (genes encoding enzymes that convert pyruvate via acetolactate to acetoin) and *acsA* (degradation of acetate), and the downregulation of *pdhABCD* (encoding the pyruvate dehydrogenase complex) (see Figs. 5 and 6 and Fig. S6 of the Supplementary Data). In this manner, the cell may diminish both the possible toxic effects caused by acetate accumulation and the acidification of the cytosol by an increased production of acetoin.

Analysis of the transcriptomic data clearly revealed that weak organic acids induce (distinct & opposite) changes in expression of genes and gene groups involved in the cell envelope (Table 2 and Fig. 6). In cells stressed with KS a clear induction of the FapR and BkdR regulons were observed, which might lead to longer and more branched acyl-lipid chains. Remarkably, acetic acid stress reduced the expression of fatty acid biosynthesis genes. The downregulation of the *des* gene, encoding the sole membrane-bound acyl-lipid desaturase, in CCCP- and KAc-treated cells indicates a possible decrease in the fluidity of the membrane by lowering the amount of unsaturated fatty acids in the membrane (Fig. S3 and Fig. 6). This gene is regulated by two-component system DesK/DesR and induced by cold-shock (Mansilla and de Mendoza, 2005). DesK is thought to sense the membrane fluidity (Cybulski et al., 2002). Sorbic acid did not alter the expression of this gene. The question whether a *des* mutant shows increased

tolerance to weak organic acid stress still remains to be answered. Several studies report on the changes in membrane composition upon weak organic acid stress, implying a possible resistance mechanism (Golden et al., 1994; Henriques et al., 1997; Ter Beek et al., 2008; Warth, 1989). The indication of plasma membrane remodeling in *B. subtilis* by KS was supported by the reduced sensitivity toward the fatty acid biosynthesis inhibitor cerulenin upon sorbic acid stress (Ter Beek et al., 2008). Cells respond to the addition of cerulenin with the induction of the FapR regulon (Schujman et al., 2003).

Further responses indicating adaptations in the cell envelope were the downregulation of genes mediated by SigX (by KS and KAc), SigW (by KS and CCC), and YvrH (only by KS). Extracytoplasmic function sigma factor SigX is involved in the regulation of cell surface modification as a defense against cationic antimicrobial peptides (Cao and Helmann, 2004). The observed downregulation of SigX-regulated genes may therefore lead to an altered (presumably more negatively-charged) cell envelope composition. This will likely repel the anion more strongly.

Our results suggest that organic acid-treated cells adapt their cell envelope. Although there is overlap between the observed cell envelope-related responses, each acid seems to induce a unique profile (Table 2). Notable, several (multiple) phospholipid biosynthesis mutants also caused induction of genes regulated by SigM and repression of the YvrH and FapR regulon (Salzberg and Helmann, 2008). Experiments with *B. subtilis* cells stressed with sorbic acid do show changes in the lipid composition (our unpublished results).

Notable, the unknown genes *yhbJj-yhcAB* were highly upregulated in KS-stressed cells and highly downregulated in KAc-stressed cells (Fig. S2). A similar reversed pattern in gene expression was observed for the genes regulated by the fatty acid and phospholipid regulator FapR (Table 2 and Fig. 6). The *yhbJ* gene encodes a possible regulator of the MarR family, and *yhcB* a trp repressor binding protein. Interestingly, *yhcA* codes for a possible multidrug resistance protein. It was shown before that the inactivation of *yhcA* led to a sorbic acid resistant phenotype (Ter Beek et al., 2008). Other transcriptome studies revealed that the *yhbJj-yhcABC* genes were highly upregulated in benzoate-adapted cells and when fully adapted *B. subtilis* cultures grown at lower pH (6 or 7) were compared to cultures grown at higher pH (7 or 9) (Kitko et al., 2009; Wilks et al., 2009). It should be noted however, that these experiments were performed in rich medium, which contains various (weak) organic acids that can cause additional effects. In addition, *B. cereus* cells stressed with HCl, lactic acid, and acetic acid, and outgrowing spores in the presence of sorbic acid also induced *yhcA* (Mols et al., 2010a, 2010b; van Melis et al., 2011).

Interestingly, CCCP stress induced the gene *sodA*, encoding superoxide dismutase (Fig. 6 and Fig. S7). This gene is thought to be regulated by SigB (Petersohn et al., 2001). Piper (1999) showed that weak organic acid enhanced the production of ROS in *S. cerevisiae*. Low pH stress in *B. cereus* also caused ROS and the induction of superoxide dismutase (Mols et al., 2010b). Whether a *sodA* mutant increases the sensitivity towards weak organic acids in *B. subtilis* remains to be elucidated.

## 5. Conclusions

Our data corroborates the notion that the effect of weak organic acids depends on the undissociated acid's level and their membrane solubility. We show that all three stresses induced responses normally seen upon nutrient limitation, indicating adaptation mechanisms against uncoupling of the proton gradient. By expressing a pH-sensitive derivative of GFP in *B. subtilis* cells, we show that all three weak acids cause acidification of the cytosol. More specifically, CCCP stress causes the largest drop in  $pH_i$ , which

supports the observed induction of both the GSR and SigM-mediated response only in CCCP-stressed cells. Interestingly, all three acids clearly induced (distinct) responses that point towards a change in the composition of the cell envelope. Our comparative study gives insight in the generic and specific transcriptional responses caused by weak acids, supporting the observed differences in growth inhibitory efficacy. The data may be used to generate input for models that provide risk assessment of food spoilage. Specific transcription profiles allow for the estimation of the likelihood of stress cross-tolerance and hence the generation of strains that are able to withstand many antimicrobial treatments and thus with the capability to become 'super spoilers'.

The described approach illustrates the value of mechanistic 'omics' data for hazard characterization where it concerns the generation of population based data on transcriptional responses towards antimicrobial compounds used as food preservatives. The data provides input for mechanistic analysis of highly stress resistant 'super spoilers' (see also Ter Beek and Brul (2010)) as well as microbes that challenge microbiological food safety (Brul et al., 2012; McMeekin et al., 2010). The limitation of the study is that such approaches yield no molecular physiological information on those organisms that become refractive to weak organic acid stress because they shut down cellular metabolism and become essentially dormant 'persister(-like)' cells (see Zhang (2014) for a recent review on stress resistant persisters). In order to capture such generally low frequent events, the use of single cell analysis techniques is imperative. Recently Pandey et al. (2013) have developed live-imaging techniques that are now extended to allow for the visualization at single cell level of fluorescent reporter proteins useful for pH<sub>i</sub> imaging as well as the action of specific stress response routes, in vegetative cells and germinating *B. subtilis* spores (Pandey et al. our unpublished observations; see for a broad overview of the use of fluorescence microscopy to study intracellular signaling in bacteria e.g. Kentner and Sourjik (2010)). Upon identification of key regulatory units in stress response reactions, like for instance the SigB regulon, appropriate (multiple) fluorescent constructs can be generated to follow cellular stress responses (see also Hornstra et al. (2009)). Combining these approaches with appropriate direct molecular sample analysis techniques in the food chain (Sowmya et al., 2012) as well as microfluidics analysis (see e.g. Fritsch et al. (2013) for a possible experimental approach), will allow for the generation of single cell molecular mechanistic data of relevance to the food chain. Due to the mechanistic basis, this data should yield potentially highly robust models of use to microbiological risk assessment. Such molecular genomics based approaches to the benefit of food production are currently being evaluated in the framework of formal microbiological risk assessment by the European Food Safety Authority (Pielat et al., 2013).

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.fm.2014.02.013>.

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