Chapter 4

Comparative Physiological and Transcriptional Analysis of Weak Organic Acid Stress in *Bacillus subtilis*

Manuscript in preparation:
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4.1. Abstract
We performed a comparative physiological and transcriptional analysis of stress responses caused by the commonly used food preservatives sorbic- and acetic acid, as well as the uncoupler carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) in exponentially grown *Bacillus subtilis*. The concentration of each acid needed to cause a similar reduction of the growth rate negatively correlated with their membrane solubility and positively correlated with the concentration of undissociated acid present. The contribution of the undissociated form of sorbic acid to the growth inhibition was the highest out of the three stresses. The anions also contributed to growth inhibition, although to a lesser extent than the undissociated form. The effect seemed most pronounced for the anion of CCCP. Time-resolved microarray analysis of sub-lethally stressed *B. subtilis* revealed that weak organic acids activate transcriptional programs normally seen upon nutrient limitation, indicated by the derepression of CcpA-regulated genes and the induction of the stringent response. Moreover, all three stresses induced diverse responses that indicate an adaptation of the cell envelope. Potassium sorbate (KS) specifically activated the FapR and BkdR regulons. On the other hand, potassium acetate (KAc) repressed the genes regulated by FapR. Interestingly, both CCCP and KAc repressed the *des* gene, encoding the sole membrane-bound acyl-lipid desaturase. The stress-specific repression of the YvrH, SigW, and SigX regulons, involved in the cell envelope, was also observed. The general stress response was induced by KAc and CCCP, but not by KS. Solely CCCP activated the expression of genes regulated by SigM and *sodA*, encoding superoxide dismutase. Finally, a clear metabolic shift to acetate utilization was seen in KAc-stressed cells: the strong induction of the *alsSD* genes, involved in the production of acetoin, and acetyl-CoA synthetase *acsA*, and correspondingly, a clear repression of the *pdhABCD* genes, encoding pyruvate dehydrogenase complex. In general, the responses seen in weak organic acid-stressed cells indicate changes in the composition of the cell envelope and adaptation mechanisms against uncoupling of the proton gradient.
4.2. Introduction

Weak organic acids 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 and inhibits the growth of spoilage bacteria, yeasts, and moulds (Beales, 2004, Brul & Coote, 1999, Davidson, 2001, Piper et al., 2001). The most active among them are: acetic, lactic, propionic, sorbic, and benzoic acid. The antimicrobial activity of this class of food preservatives depends on the pKₐ 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. After entry into the cell, a new equilibrium is formed between both forms. The resulting release of protons lowers the proton gradient and may cause lowering of the intracellular pH. The latter will affect virtually all biochemical processes, including the redox state, DNA transcription, protein synthesis and folding, enzyme activities, and transport over the membrane (Beck & Jahns, 1996, Cotter & Hill, 2003, Foster, 2004, Veine et al., 1998). The uncoupling effect and the possible acidification of the cytosol are thought to be the main modes of action of weak organic acids (Beales, 2004, Brul & Coote, 1999, Davidson, 2001, Piper et al., 2001). Furthermore, the released anion is considered to be responsible for a rise in osmolarity and, depending on the specific preservative used, to affect cytosolic enzymes (Azukas et al., 1961, Russell, 1992, York & Vaughn, 1964). 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, Krebs et al., 1983, Sheu & Freese, 1972, Stratford & Anslow, 1998). The hydrophobicity of the weak acid likely relates to its membrane disrupting effect. Although the general modes of action are established, differences between the effectiveness of weak organic acids are observed. For instance, the pKₐ values of sorbic and acetic acid are similar (Table 4.1), however in general more acetic acid is needed to obtain similar growth-inhibitory effects (e.g. Abbott et al., 2007, Piper et al., 1998). This can be partly explained by the obvious diversity in the chemical properties of these two preservatives (Table 4.1), and the differences in the ability of the organism to metabolize the acid.

Depending on the challenged organism and the weak acid used, various resistance mechanisms have been described. In general, ATPases in the membrane are thought to pump out excess protons at the cost of ATP to restore pH homeostasis (Beales, 2004, Brul & Coote, 1999, Davidson, 2001, Piper et al., 2001). To prevent the accumulation and toxic effects of the anion, Saccharomyces cerevisiae induces the Pdr12p pump to extrude the sorbate anion at the cost of ATP (Holyoak et al., 1999). Remarkably, it has been reported recently that this pump also recognizes the uncoupler carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) as a substrate (Hendrych et al., 2008). In addition, some organisms metabolize the acid in order to minimize the effects of the anion. For instance,
Table 4.1. Properties of the weak organic acids compared in this study.

<table>
<thead>
<tr>
<th>Weak organic acid</th>
<th>Chemical structure</th>
<th>pK\textsubscript{a}</th>
<th>Log K\textsubscript{ow\textsuperscript{a}}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorbic acid</td>
<td><img src="image" alt="Sorbic acid structure" /></td>
<td>4.76</td>
<td>1.33</td>
</tr>
<tr>
<td>Acetic acid</td>
<td><img src="image" alt="Acetic acid structure" /></td>
<td>4.76</td>
<td>-0.17</td>
</tr>
<tr>
<td>CCCP</td>
<td><img src="image" alt="CCCP structure" /></td>
<td>5.95</td>
<td>3.38</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Experimentally determined octanol-water partition coefficient (log K\textsubscript{ow}) values were taken from the KowWin-Demo-Database (http://www.srcinc.com/what-we-do/databaseforms.aspx?id=385).

Zygosaccharomyces 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). Several studies report on the changes in membrane composition upon weak organic acid stress, implying a possible resistance mechanism. Changes in fatty acid composition in sorbate-stressed Z. rouxii cells have been observed (Golden et al., 1994) and suggested in Bacillus subtilis by us (Ter Beek et al., 2008; see Chapter 2). Benzoate-adapted S. cerevisiae and Z. Bailii cells reduced their permeabilities to benzoic acid (Henriques et al., 1997, Warth, 1989). Recently, it was shown that S. cerevisiae possesses an aquaglyceroporin (Fps1) that also facilitates the diffusion of undissociated acetic acid into the cell. Upon stress, Fps1 is degraded, thereby increasing the tolerance to acetic acid stress (Mollapour & Piper, 2007).

Weak acid resistance mechanisms in bacteria are largely unknown. Depending on the species however, bacteria can induce several systems to counteract a drop in the internal pH when encountering (extreme) low pH stress. The general stress response (GSR) (SigB and RpoS-mediated) is induced upon low pH stress (Bearson et al., 1997, Hecker & Volker, 2001). The SigM regulon, involved in maintaining membrane and cell wall integrity, is also induced in B. subtilis at low pH (Thackray & Moir, 2003). Furthermore, the induction of proton pumps, chaperones, and the production of basic compounds by urease, decarboxylases, and deiminase are known factors contributing to acid tolerance (Bearson et al., 1997, Cotter & Hill, 2003). Although the importance of low pH stress response systems in weak-acid resistance
development remains unclear, it has been shown that the acid tolerance responses of *Salmonella typhimurium* and *Escherichia coli* increased the resistance to weak organic acids (Baik *et al.*, 1996).

To reveal the generic and specific responses of weak organic acids, microarray technology is a powerful tool. Comparative microarray studies of *S. cerevisiae*, grown anaerobically in chemostats, have shown that compounds with similar hydrophobicities activate overlapping responses (Abbott *et al.*, 2007). The more lipophilic compounds benzoate and sorbate induced responses more related to the cell wall, while the responses induced by the less lipophilic compounds acetate and propionate focused on membrane-associated transport processes (Abbott *et al.*, 2007). Gram-negative bacterium *E. coli* was subjected to acetate, propionate, and also CCCP (Polen *et al.*, 2003). Short-term exposure to these three stresses all induced the RpoS-mediated GSR. Interestingly, a proteome study on *E. coli* also revealed the induction of the GSR by acetate; however, formate (pKₐ value of 3.75) repressed the RpoS-mediated genes (Kirkpatrick *et al.*, 2001). Recently, we have shown that *B. subtilis* mildly stressed with potassium sorbate did not induce the SigB-mediated GSR (Ter Beek *et al.*, 2008). Only severe inhibition of growth (71%) induced the GSR. Since neither the viability nor the growth rate in a *sigB* mutant was affected, it was concluded that the GSR is not the key response of cells encountering sorbic acid stress (Ter Beek *et al.*, 2008).

Bacilli are common food spoilers. They can form highly-resistant endospores able to survive the food processing steps. In particular, the germination and outgrowth of these spores pose a threat. Here we report the time-resolved transcriptional responses of model organism *B. subtilis* subjected to potassium acetate (KAc) and also CCCP, a powerful uncoupler and weak organic acid. We performed a thorough comparison of the transcriptional responses caused by KAc, CCCP and the recently published data on sorbic acid stress (Ter Beek *et al.*, 2008). We determined the minimal generic transcriptional response to weak organic acids, as well as the specific and opposite responses involved. We show that all three weak organic acids induced responses normally seen upon nutrient limitation, indicating adaptation mechanisms against uncoupling of the proton gradient. Interestingly, all three acids clearly induced (distinct) responses that point towards a change in the composition of the cell envelope.

### 4.3. Materials & Methods

#### 4.3.1. Bacterial strain, growth and stress conditions

168 wild-type (WT) *B. subtilis* strain PB2 was cultivated in a defined minimal medium as described previously (Ter Beek *et al.*, 2008). The medium was buffered with 80 mM 3-[N-morpholino]propanesulfonic acid 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. Strain PB2 was grown exponentially, transferred to a SpectroMax Plus microtitre plate reader...
(Molecular Devices Corp.) at an optical density at 600 nm (OD\textsubscript{600}) of 0.08 (which corresponds to an OD\textsubscript{600} of 0.2 in a 1 cm path length spectrophotometer) and stressed with various concentrations of KAc (5 – 250 mM) or CCCP (0.25 – 10 µM). Additional stress experiments were performed to test for synergy in antimicrobial action by using different combinations of 3 mM KS, 25 mM KAc, 0.85 µM CCCP, 0.5 M NaCl, and 5 µg/ml cerulenin. Cells were further cultivated in the microtitre 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 110 min of the control (no addition of stress) and the stressed culture. All conditions were tested in the microtitre plate reader at least in triplicate and biologically independent experiments were performed at least twice. The concentration of each weak acid required to cause a growth inhibition of 50% (IC\textsubscript{50}) was determined and the relative IC\textsubscript{50} was calculated from the IC\textsubscript{50} value obtained at pH 6.4 (IC\textsubscript{50} at tested pH value / IC\textsubscript{50} at pH 6.4).

4.3.2. Time-resolved transcriptome analysis using DNA microarrays

An exponentially growing culture of \textit{B. subtilis} WT strain PB2 was split in two and cultured in well-controlled batch-fermentors as described previously (Ter Beek \textit{et al.}, 2008). At an OD\textsubscript{600} of 0.2, one culture was stressed with 25 mM KAc or 0.85 µM 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 \textit{et al.} (2008). Total RNA was isolated as described previously (Keijser \textit{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 \textit{et al.} (2008). The data was normalized using J-Express Pro 2.7 software (MolMine AS). Genes with more than one missing value in the time-series were omitted. After the processing of the microarray data 3,746 and 3,685 genes remained for each time-point, of which 503 and 582 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 \textit{P} value was below 0.01.

The microarray data was analyzed using two complementary methods. Hierarchical clustering (Eisen \textit{et al.}, 1998) of specific sub-sets 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 \textit{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 \textit{et al.}
Comparative Analysis of Weak Organic Acid Stress (2008). Generally, absolute T values around 4.1 and higher (depending on the group size) are significant.

4.4. Results & Discussion

4.4.1. Comparison of the growth inhibitions caused by weak organic acids reveals differences in the contribution of the form of each acid

In this study, we compared the effects of weak organic acids. Besides the commonly used food preservatives sorbic- and acetic- acid, we also investigated the powerful uncoupler CCCP. Although the side chains of sorbic- and acetic- acid differ significantly, they have a similar pKₐ of 4.76 (Table 4.1). CCCP, which is also a weak organic acid, has a pKₐ value of 5.95. Comparing the octanol-water partition coefficient (log Kₐ) values shows that CCCP is the most lipophilic compound and acetic acid the least. 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 (S⁻) also contributed to the growth inhibition although to a much lesser extent. Using the same approach, we determined the pH dependence of the action of acetic acid and the uncoupler CCCP on exponentially growing *B. subtilis* cells in a defined minimal medium. Increasing concentrations of either potassium acetate (KAc) or CCCP resulted in a decreasing growth rate when grown at pH 6.4 (Fig. 4.1A and B). Note that much lower concentrations of CCCP (µM range) are needed to obtain a similar inhibition of growth than for KAc (mM range). Similar qualitative trends of the growth curves were observed for all pH values tested, although higher concentrations were needed at higher pH to obtain similar reductions in OD₆₀₀ (our unpublished data). We plotted the percentages of growth inhibition (GI %) against the concentrations of the anion (Ac⁻ and CCCP⁻) and the undissociated (HAc and HCCCP) form of the acids (Fig. 4.1C, D, E and 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 CCCP. This clearly demonstrates that Ac⁻ and CCCP⁻ also contribute to the growth inhibition. Interestingly, low concentrations of KAc (5 and 10 mM) at pH 7.8 revealed a small but significant increase in the growth rate (Fig. 4.1C).

We determined the concentration of each weak acid needed to cause a growth inhibition of 50% (IC₅₀) for each pH from the obtained growth inhibition curves (Table 4.2). It can be clearly seen that with increasing pH higher concentrations of total acid (KS, KAc, or CCCP) were required to obtain similar IC₅₀ values. This supports the notion that the effect of weak organic acids is mainly caused by the undissociated form. On the other hand, the calculated amount of undissociated acid present in the medium decreased with increasing extracellular pH, indicating that growth inhibition is not solely caused by the undissociated form of the acid. Nevertheless, the total concentration needed to achieve a similar reduction in the growth rate
Fig. 4.1. 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 µM (open diamonds), 0.5 µM (closed triangles), 1 µM (open triangles), 1.5 µM (closed squares), 2.25 µM (open squares), and 3 µM (crosses) CCCP. The OD₆₀₀ 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 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.
### Table 4.2. IC\textsubscript{50} calculated for the total-, undissociated acid-, and anion- concentration of each weak organic acid at different pH values.\textsuperscript{a}

<table>
<thead>
<tr>
<th>pH</th>
<th>total IC\textsubscript{50} (mM)</th>
<th>undissociated acid IC\textsubscript{50} (µM)</th>
<th>anion IC\textsubscript{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.9</td>
<td>3.2 ± 0.1</td>
<td>27.6 ± 1.7</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>6.4</td>
<td>8.1 ± 0.2</td>
<td>53.8 ± 3.8</td>
<td>1.31 ± 0.08</td>
</tr>
<tr>
<td>7.4</td>
<td>72.7 ± 3.4</td>
<td>231.1 ± 15.6</td>
<td>4.13 ± 0.67</td>
</tr>
<tr>
<td>7.8</td>
<td>112.9 ± 5.1</td>
<td>356.4 ± 19.6</td>
<td>5.23 ± 0.33</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The concentrations causing 50% of growth inhibition (IC\textsubscript{50}) are based on the data presented in Fig. 4.1 and 4.2 and Fig. 2.1 from Chapter 2 (Ter Beek et al., 2008). Undissociated acid- and anion-concentrations were calculated from the total concentration using the Henderson-Hassellbalch equation. Values shown represent the means of minimally four biologically independent experiments each consisting of four technical replicates, including the standard errors.

negatively correlated with the log K\textsubscript{ow} values of each weak organic acid (Table 4.1 and 4.2), indicating that their solubility into the membrane has a prominent effect on the growth inhibition or is the rate-limiting step. To be able to better compare the effects of the weak acids we calculated the IC\textsubscript{50} relative to the value at pH 6.4 (Fig. 4.2). In theory, when the inhibitory effect is caused by only one form of the acid (dissociated or undissociated), the relative IC\textsubscript{50} value remains 1 at every pH value. Therefore, comparing the three tested compounds, HS contributed the most (smallest deviation from 1) to the observed GI % and consequently S\textsuperscript{−} the least (largest deviation from 1). The contribution of the anion to the GI % seemed to be most prominent for CCCP\textsuperscript{−} and slightly less for Ac\textsuperscript{−} (Fig. 4.2C). 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\textsuperscript{−}. Finally, considering the size of acetate, Ac\textsuperscript{−} might be able to enter the cell through a membrane channel.

4.4.2. Overall transcriptional responses of B. subtilis sub-lethally stressed with weak organic acids

We showed that different amounts of weak acids were needed to obtain a similar reduction of the growth rate and that these concentrations negatively correlated with the solubility into the membrane (represented by the log K\textsubscript{ow} values in Table 4.1). However, the contributions of the different forms of the acid seemed to differ depending on the weak organic acid used (Fig. 4.2). We set out not only to elucidate the minimal generic transcriptional response to weak organic acids, but also to identify specific responses. Using time-resolved microarray technology we analyzed the transcriptional responses of _B. subtilis_...
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Relative IC₅₀ values reveal differences in the contribution of the form of the weak organic acid molecule. The relative IC₅₀ was calculated from the IC₅₀ value obtained at pH 6.4 for (A) the total concentration, (B) the undissociated-, and (C) the dissociated form of the acid (IC₅₀ at tested pH value / IC₅₀ at pH 6.4). Note the differences in the scale of the y-axis. The error bars indicate the standard errors.

Sub-lethally stressed with weak organic acids. *B. subtilis* cells were grown to early exponential-phase at pH 6.4 in well-controlled batch fermentors and treated with 25 mM KAc or 0.85 µM 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 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.

All expression data were analyzed and compared using T-profiler, which transforms the transcriptional data of single genes into the behaviour 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 caused by at least one stress are presented in Appendix C (see Tables S1 – S4 for a full quantitative overview of the gene-regulation profiles upon stress application). The treatment of *B. subtilis* cells with KAc or CCCP resulted in the significantly different expression of 503 and 582 genes, respectively, in comparison with the control. The significantly regulated genes, including the 459 differentially expressed genes in the KS treatment (Ter Beek *et al.*, 2008) were divided into 7 categories and analyzed with hierarchical clustering (Eisen *et al.*, 1998). These categories consisted of genes uniquely regulated by (1) KS, (2) KAc, (3) CCCP, (4) KS and KAc, (5) KAc and CCCP, (6) CCCP and KS, or by (7) KS, KAc and CCCP (Fig. 4.3, 4.4, and S1 – S6 in Appendix C). In total, 1,107...
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Fig. 4.3. Venn diagrams showing the number of significantly regulated genes unique 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 upgerulated by KS and CCCP (125+63+33+35+31+224=511 genes), etc.

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

Ninety-six out of the 1,107 differentially expressed genes were uniquely induced or repressed by all three acids. 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. 4.3 and 4.4). Accordingly, 12 genes showed opposite expression within the expression patterns of the three weak organic acids.

Interestingly, major changes in genes involved in carbon metabolism were observed (Fig. 4.4). Gene groups involved in carbohydrate metabolism and TCA cycle were induced. 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). The utilization of acetate can provide a source of energy and might relieve the cell from anion accumulation, especially in the case of KAc stress. T-profiler analysis also revealed the significant derepression of CcpA-regulated genes (Fig. 4.5A), which normally occurs when the preferred carbon source
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 Euclidean 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 colourimetrically, 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.
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(glucose, fructose, and mannose) is depleted (Deutscher et al., 2002). The significant downregulation of gene groups involved in nucleotide metabolism and the RelA-dependent stringent control (Fig. 4.5D and E), as well as protein synthesis and translation (Tables S3 and S4 in Appendix C) also indicated a possible energy and/or nutrient limitation experienced by the cell and correlated with the observed (~30%) reduction in growth.

Notable is the induction of the ureABC operon, which codes for urease (Cruz-Ramos et al., 1997) (Fig. 4.4). However, as demonstrated before, a urease mutant did not show an altered phenotype upon KS stress when compared to the WT (Ter Beek et al., 2008). Most of the arginine biosynthesis and transport genes were significantly downregulated by all three stresses (Fig. 4.4). KS and KAc stress did reveal a significant repression of genes controlled by AhrC, the arginine metabolism regulator (North et al., 1989) (Fig. 4.5F). In addition, KAc also induced a short (at t = 10 min), but significant derepression of AhrC-regulated genes. No T values could be calculated for CCCP-stressed cells, since filtering of the CCCP microarray data resulted in an AhrC-regulated gene group with less than seven genes. T-profiler requires at least 7 genes in a group in order to calculate reliable T values (E < 0.05) (Boorsma et al., 2005). Most genes of the KipR regulon (Wang et al., 1997) were also found to be repressed by weak organic acid stress (Fig. 4.4 and 4.5F). No T values could be calculated for KipR-regulated genes upon CCCP stress, as there were fewer than 7 expressed genes in that group. However, hypergeometric distribution analysis of the response to CCCP revealed an enrichment of the genes negatively regulated by AhrC (P < 0.0001) and KipR (P < 0.0001). 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). The derepression of the KipR regulon may indicate the release of the “brake” on the sporulation-regulatory cascade. We have shown previously that the transcriptome of KS-treated cells did not reveal an induction of sporulation and no increase in spore counts were detected (Ter Beek et al., 2008). On the contrary, preliminary results show that KS stress during exponential growth delayed sporulation induced in the stationary phase (Hornstra et al., 2009, in press; see Chapter 6). As in KS-treated cells, T-profiler did reveal a significant downregulation of genes repressed by sporulation master regulator Spo0A in a KAc-treated culture (Table S1 in Appendix C). However, the downregulation of the arginine biosynthesis genes negatively controlled by AhrC and positively controlled by transition-state regulator AbrB may explain the observed response. Although some genes involved in sporulation were significantly regulated by all three weak organic acids, T-profiler did not reveal any further significant regulation of gene groups involved in sporulation (our unpublished data). Most of the early sporulation genes significantly induced by the stresses can be explained by their SigH-mediated regulation. In untreated cells the SigH regulon becomes active at the end of exponential growth preceding competence and sporulation (Haldenwang, 1995). Although the trends of the T values are similar, only KS induced a short significant induction of the genes regulated by SigH (Table S2 in Appendix C).
Fig. 4.5. Weak organic acids induce adaptation mechanisms against uncoupling of the proton gradient. The $T$ values of the following gene groups are shown: (A) negatively regulated by CcpA, (B) carbon metabolism, (C) citrate cycle (TCA) cycle, (D) nucleotide metabolism, (E) positive and negative stringent control (RelA-dependent), and (F) negatively regulated by KipR and AhrC. Groups of genes that are negatively regulated by the transcription factor mentioned are indicated by the minus symbol. The $T$ values presented were 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 caused by KS (diamonds), KAc (triangles) and CCCP (squares). The gene group of the positive stringent control in CCCP-stressed cells (dashed lines) did not show significant $T$ values in the time course analyzed.
Remarkably, most of the unknown genes yxiF-yxzC-yxiGH-yxzG-yxiIJKL that lay adjacently to each other in the genome were significantly downregulated by all three weak organic acids (Fig. 4.4). Interestingly also salicylic acid, another weak organic acid with pK_a of 3, repressed most of these unknown genes (Duy et al., 2007). The function of these genes in response to weak organic acid stress remains to be elucidated.

In summary, all three weak organic acids induced responses that are generally observed when nutrients become limiting. This may indicate the induction of adaptation mechanisms against a possible energy/nutrient limitation, which can result from uncoupling of the proton gradient. However, no induction of sporulation was observed. No apparent similar responses involved in the maintenance of pH homeostasis were identified (further discussed below).

4.4.4. Specific-, opposite- and co-responses to KS, KAc, and CCCP

Although the transcriptional response to sorbic acid has been extensively described (Ter Beek et al., 2008), it is also relevant to discuss the responses that are unique to KS alone or similar to one of the other compounds. The majority of genes regulated by each of the three weak organic acids were unique for each stress, ranging between 48.8 and 69.3% for the upregulated genes and 47.3 and 57.7% for the downregulated genes (Fig. 4.3). This indicates that the way the cell experiences these stresses is more specific than similar, although all three chemicals are weak organic acids. However, hypergeometric distribution analysis showed that the overlap of genes by each possible combination of stress was highly enriched (for all: \( P < 0.0001 \)), which indicates the three stresses induced responses that have a lot in common. Compared to each other, CCCP induced (69.3%) and repressed (57.7%) more genes uniquely, than KS or KAc did. There largest overlap of induced genes was seen in KS and KAc stressed cultures (14.5%). However, the downregulated genes showed more overlap between the CCCP- and KAc- (11.1%), and the KAc- and KS-treated cells (9.2%), than between the KS- and CCCP-stressed cultures (4.7%).

4.4.5. Weak organic acids induce (distinct) adaptations that further indicate changes in carbon metabolism

Among the 56 genes uniquely regulated by CCCP and KS, 11 genes were derepressed by CcpA, including the levanase operon levDEFG-sacC, involved in fructose and levanan metabolism (Fig. S3 in Appendix C). This operon is regulated by the levanase regulator LevR and SigL, involved in alternative carbon and nitrogen metabolism (Debarbouille et al., 1991, Martin-Verstraete et al., 1995). CcpA has been shown to regulate the expression of SigL (Choi & Saier, 2005). Significant \( T \) values for the SigL-mediated gene group were found in KS- and CCCP-stressed cells (Fig. 4.6A). Although KAc caused a significant derepression of CcpA, the levanase operon and the SigL regulon were not induced (Fig. 4.5A). Only CCCP
Fig. 4.6. Weak organic acids induce (distinct) responses that further indicate changes in carbon metabolism. The $T$ values of the following gene groups are shown: (A) SigL, (B) negatively regulated by IolR, (C) AbrB, and (D) negatively regulated by CodY. Depicted are the profiles caused by KS (diamonds), KAc (triangles) and CCCP (squares). A distinction is made between groups of genes that are positively or negatively regulated by the transcription factor mentioned (indicated by the plus and minus symbol, respectively). See the legend of Fig. 4.5 for further details.

repressed the gene group positively regulated by CcpA (Table S1 in Appendix C). The strong derepression of the genes negatively regulated by CcpA in CCCP-treated cells may explain this observation (Fig. 4.5A) and suggests differences in the promoter-strength between the positively- and negatively-CcpA regulated genes. The same explanation may be valid for the observed induction of the whole $iol$ operon, responsible for myo-inositol catabolism and regulated by the IolR repressor and negatively by CcpA (Yoshida et al., 2008), only in a CCCP-stressed culture (Fig. 4.6B and Fig. S6 in Appendix C). Surprisingly, KAc also induced the gene group positively regulated by CcpA (Table S1 in Appendix C). However, the strong induction of the $alsSD$ genes seen only in KAc-treated cells may explain the observed induction. The $alsSD$ genes are known to be induced by acetate as well as in the post-exponential phase (Renna et al., 1993). These genes code for enzymes that convert pyruvate via acetolactate to acetoin and are regulated by, besides CcpA, AlsR (Renna et al., 1993).
Simultaneously with the strong induction of the \textit{alsSD} genes we observed solely in KAc-stressed cells the significant repression of the \textit{pdhABCD} genes, encoding the pyruvate dehydrogenase complex (Fig. S5 in Appendix C). In CCCP- and KAc-treated cell we also discovered the significant downregulation of \textit{ldh (lctE)} coding for lactate dehydrogenase (Fig. S2 in Appendix C). As a consequence, the conversion of pyruvate to acetyl-CoA and lactate is minimized and shifted to non acidic acetoin production. Noteworthy, an \textit{alsS} mutant acidifies the medium more rapidly than the WT (Kinsinger \textit{et al.}, 2005). This together with the earlier discussed significant induction of \textit{acsA} (degradation of acetate) and the \textit{acuABC} genes (activation of AcsA) may reflect a metabolic switch to alleviate the cell from high internal concentrations of acetate and provide acetyl-CoA for the TCA-cycle via an alternative route.

KS and KAc uniquely induced 14.5% of all upregulated genes (Fig. 4.3). This was the largest overlap seen among the three stresses. Hierarchical clustering of the genes significantly regulated solely by KS and KAc revealed many genes derepressed by transition-state regulator AbrB and early-stationary-phase regulator CodY (Sonenshein, 2005, Strauch & Hoch, 1993) (Fig. S1 in Appendix C). These responses may indicate that nutrients have become limiting. After a short induction of the gene group positively regulated by AbrB caused by solely KAc, a clear repression of this gene group was observed in both KS- and KAc-stressed cells (Fig. 4.6C). Noteworthy, the AbrB-mediated response was slower in KAc-treated cells and was not induced by CCCP. On the other hand, compared to KS and KAc, CCCP induced a short opposite CodY-mediated response (Fig. 4.6D).

In conclusion, the generic trait of weak organic acid-stressed cells points towards major changes in carbon metabolism.

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

Sorbic acid caused a clear induction of the fatty acid biosynthesis genes regulated by the fatty acid and phospholipid regulator FapR (Schujman \textit{et al.}, 2003) (Fig. 4.7A). The gene group regulated by BkdR, involved in the synthesis of precursor molecules for branched-chain fatty acids (Debarbouille \textit{et al.}, 1999), was also uniquely induced by KS stress (Table S1 in Appendix C). 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 \textit{et al.}, 2002, Schujman \textit{et al.}, 2003). The induction of plasma membrane remodelling in \textit{B. subtilis} by KS was supported by the reduced sensitivity toward the fatty acid biosynthesis inhibitor cerulenin (CL) upon sorbic acid stress (Ter Beek \textit{et al.}, 2008). Cells respond to the addition of CL with the induction of the FapR regulon (Schujman \textit{et al.}, 2003). Remarkably, KAc caused a significant repression of the fatty acid biosynthesis genes, which could in theory result in shorter acyl-lipids in the membrane (Fig. 4.7A). CCCP induced only two genes (\textit{fabHB} and
Fig. 4.7. Weak organic acids induce (distinct) adaptations of the cell envelope. Log₂ ratios of (A) the significantly expressed genes of the FapR regulon, and (B) the significantly expressed yhbIJ-yhcAB genes. Expression caused by KS, KAc and CCCP is shown in blue, red and green, respectively. See the legends for details on the individual genes. The T values of the following gene groups are shown: (C) SigM and SigW, and (D) SigX and negatively regulated by YvrH. Depicted are the profiles caused by KS (diamonds), KAc (triangles) and CCCP (squares). Groups of genes that are negatively regulated by the transcription factor mentioned are indicated by the minus symbol. See the legend of Fig. 4.5 for further details. T values for the gene groups that are negatively regulated by FapR (YlpC) (Schujman et al., 2003) and SigM (Jervis et al., 2007) were manually calculated, since the current version of T-profiler does not contain these gene groups.

yhfC) of the FapR regulon significantly, but also repressed the gene fabF. Opposite regulation of the transcription factor gene groups in KS- and KAc-treated cells was only seen for the FapR regulon (Table S1 in Appendix C). Intriguingly, the unknown genes yhbIJ-yhcAB also illustrated the same reverse pattern in gene expression upon KS and KAc stress (Fig. 4.7B and S1 in Appendix C). In addition, CCCP only induced significantly yhbJ. The yhbl 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). Because of the similar expression patterns we speculate whether these unknown genes are
somehow linked to the FapR regulon. Notewort hy, a recent transcriptome study on acid and base stress revealed that the \textit{yhlJ}-\textit{yhcABC} genes were among the strongest induced genes when fully adapted \textit{B. subtilis} cultures grown at lower pH (6 or 7) were compared to cultures grown at higher pH (7 or 9) (Wilks \textit{et al.}, 2009). It should be noted however, that these experiments were performed in rich medium, which contains various organic acids that can cause additional effects.

Significant regulation of another gene further illustrated the possible change in membrane composition upon weak organic acid stress. Namely, both KAc and CCCP caused the significant downregulation of the \textit{des} gene, encoding the sole membrane-bound acyl-lipid desaturase, which is regulated by two-component system DesK/DesR and induced by cold-shock (Mansilla & de Mendoza, 2005) (Fig. S2 in Appendix C). DesK is thought to sense the membrane fluidity (Cybulski \textit{et al.}, 2002). The downregulation of \textit{des} might result in lower amounts of unsaturated fatty acids in the membrane, leading to a decrease in membrane fluidity. Sorbic acid did not alter the expression of this gene. The question whether a \textit{des} mutant shows increased tolerance to weak organic acid stress still remains to be answered.

Interestingly, we discovered altered expression of gene groups involved in the adaptation of the cell envelope. The SigM regulon in \textit{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 & Moir, 2003). The extracytoplasmic function sigma factor SigX and SigW are also involved in the regulation of cell envelope homeostasis (Helmann, 2002). CCCP clearly induced genes regulated by SigM (Fig. 4.7C). However, KS and KAc both did not induce this regulon. The induction of SigM by CCCP can be the result of cell envelope stress and/or acidification of the cytosol (further discussed below). A downregulation of SigW-mediated genes was seen in KS and CCCP-stressed cells and not in an acetate-treated culture (Fig. 4.7C). 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 (Fig. 4.7D). Extracytoplasmic function sigma factor SigX is involved in the regulation of cell surface modification as a defence against cationic antimicrobial peptides (Cao & 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. YvrH is another regulator involved in the homeostasis of the cell surface (Serizawa \textit{et al.}, 2005). Although there is overlap between the SigX regulon and that of YvrH, only KS repressed this regulon significantly (Fig. 4.7D).

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. Noteworthy, several (multiple) phospholipid biosynthesis mutants also caused induction of genes regulated by SigM and repression of the YvrH and FapR regulon (Salzberg & Helmann, 2008). Whether the observed transcriptional responses actually lead to
changes in the membrane and cell wall needs to be verified. However, initial experiments do show changes in the lipid composition of *B. subtilis* when stressed with sorbic acid (our unpublished results).

### 4.4.7. Induction of the general stress response by CCCP and KAc indicate possible ATP depletion or intracellular acidification

T-profiler analysis showed the significant induction of the SigB-mediated GSR by KAc and CCCP only (Fig. 4.8A). 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). In addition, it was shown that the induction of the GSR by nutritional stress is preceded by a drop in ATP levels (Zhang & Haldenwang, 2005). This might indicate that the observed induction of the GSR in CCCP- and KAc-treated cells is the consequence of a (severe) drop in ATP or the intracellular pH. Polen *et al.* (2003) also reported the induction of the RpoS-mediated GSR in *E. coli* upon acetate, propionate and CCCP stress. On the contrary, formate did not induce the GSR in *E. coli* (Kirkpatrick *et al.*, 2001). In addition, 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 intracellular pH levels were not severely affected. Since in our studies similar growth inhibiting concentrations (~30%) were used, this clearly shows the different modes of action of weak organic acids. The SigM regulon can also be induced by low pH (Thackray & Moir, 2003). As discussed above, CCCP induced the genes regulated by SigM (Fig. 4.7C). This is a further indication that in weak acid (CCCP) treated cells acidification takes place. Whether the concentrations of weak acids tested here actually cause an intracellular acidification and whether this correlates to the observed induction of the GSR and/or SigM-regulon remains to be elucidated (see Chapter 5).

Interestingly, CCCP stress, showing the strongest SigB-mediated response, induced the gene sodA, encoding superoxide dismutase (Fig. S6 in Appendix C). This gene is thought to be regulated by SigB (Petersohn *et al.*, 2001). Piper (1999) showed that weak organic acid enhanced the production of reactive oxygen species in *S. cerevisiae*. Yeast grown anaerobically in chemostats and stressed with weak organic acids induced SOD2, which encodes the Mn-containing mitochondrial superoxide dismutase (Abbott *et al.*, 2007). Whether a sodA mutant increases the sensitivity towards weak organic acids in *B. subtilis* remains to be elucidated. Additionaly, the unknown yvkAB genes were only induced by CCCP (Fig. S6 in Appendix C). The yvkA gene encodes a possible multidrug-efflux transporter and yvkB codes for a possible transcriptional regulator. Interestingly, these genes show homology with pqrAB, responsible for the resistance to paraquat (a reactive oxygen species generator) in *Streptomyces coelicolor* (Cho *et al.*, 2003).
Fig. 4.8. Responses caused by KAc and CCCP that may indicate an ATP depletion, acidification, and adaptations to changes in osmolarity. The T values of the following gene groups are shown: (A) SigB and (D) SigD. Depicted are the profiles caused by KS (diamonds), KAc (triangles) and CCCP (squares). See the legend of Fig. 4.5 for further details. Log2 ratios of (B) the significantly expressed \( ktrAB \) genes and (C) the significantly expressed \( opuCA-CD \) genes. Expression caused by KAc and CCCP is shown in red and green, respectively. See the legends for details on the individual genes.

4.4.8. Indications for a rise in osmolarity?

Anion accumulation inside the cell is considered to be one of the reasons for the growth inhibition caused by weak organic acids (Azukas et al., 1961, Russell, 1992, York & Vaughn, 1964). Interestingly, KAc downregulated, and CCCP upregulated the \( yuaA-yubG \) (\( ktrAB \)) operon (Fig. 4.8B). The \( ktrAB \) genes encode a high affinity \( K^+ \) uptake system and are required to sustain growth when under osmotic up-shock conditions (Holtmann et al., 2003). However, osmotic shock or the addition of \( K^+ \) in the medium did not seem to change the expression of \( ktrAB \). Cells challenged with an increase in (extracellular) osmolarity initially respond by the rapid accumulation of \( K^+ \), followed by the uptake and synthesis of compatible solutes (Bremer, 2002). The genes \( opuCA-CD \), encoding a high affinity system for the uptake of compatible solutes, surprisingly also showed opposite transcriptional profiles (Fig. 4.8C). KAc upregulated and CCCP repressed these genes. Whether anion (\( Ac^- \)) accumulation would cause an opposite response normally seen upon an extracellular osmotic up-shock is unclear.
The addition of 25 mM KAc in itself is not considered to cause osmotic stress. However, the addition of 25 mM K⁺ may have caused a change in the potassium homeostasis. Noteworthy, a study done with *E. coli* K-12 showed that the intracellular K⁺ concentrations increased upon sodium acetate stress and decreased in CCCP-treated cells (Diez-Gonzalez & Russell, 1997). Although the observed transcriptional responses involved in the adaptations to changing osmolarity are apparent, until now, it is unclear why these responses occurred or whether they are a consequence of increased anion concentrations.

4.4.9. The transcriptional response to CCCP and KAc indicate changes in motility

SigD is an alternative sigma factor that regulates the expression of genes involved in flagellar assembly, motility, chemotaxis and autolysis (Helmann & Moran, 2002). T-profiler revealed significant repression of SigD-regulated genes upon KAc and CCCP stress (Fig. 4.8D). However, CCCP seemed to induce the cell’s motility again 30 min after addition of the compound. Interestingly, hyperosmotic shock caused by NaCl also repressed the cell’s motility and induced the earlier discussed GSR (Steil et al., 2003). In addition, the repression and induction of the SigD- and SigB-regulon, respectively, was also observed in several (multiple) phospholipid biosynthesis mutants (Salzberg & Helmann, 2008).

4.4.10. Other responses

Interestingly, after a short downregulation of genes negatively regulated by the ferric uptake repressor Fur (Moore & Helmann, 2005), a clear induction was observed in KS- and KAc-stressed cells (Fig. 4.9A). Remarkably, CCCP stress caused an almost opposite Fur response. Genes regulated by Fur are not known to be regulated by any other transcription factors and mostly contain an identified SigA promoter. Therefore, the distinct profiles seen for the Fur-regulated gene group caused by CCCP on one hand and by KS and KAc on the other hand, seem to be the result of actual changes in Fur activity itself. Although the derepression indicated a possible iron-limitation, it was shown recently that increased concentrations of iron in the medium did not alter the susceptibility for sorbic acid stress (Ter Beek et al., 2008). On the other hand, cells grown at high osmolarity do experience an iron limitation and therefore induce the Fur regulon (Hoffmann et al., 2002, Steil et al., 2003). It remains to be elucidated whether increased iron concentrations or a fur mutation change the sensitivity of the cells to KAc or CCCP.

Repression of the BirA regulon was unique for CCCP stress (Fig. 4.9B). BirA represses genes involved in the synthesis of biotin, a cofactor required for numerous carboxylases, like acetyl-CoA carboxylase, needed in the initiation of fatty acid biosynthesis, and pyruvate carboxylase (*pycA*). Normally, biotin limiting conditions derepress the biotin operon and *vice versa* (Perkins & Pero, 2002). We speculate whether the specific downregulation of BirA-regulated genes might modulate the biosynthesis of fatty acids or block pyruvate from
entering the TCA cycle by inactivating PycA, shifting metabolism to acetate utilization even further.

Although CCCP and acetic acid comprise complete different membrane affinities as reflected by their octanol-water partition coefficient (Table 4.1), 31 and 46 genes were respectively up- and downregulated, by both stresses and not by KS (Fig. 4.3). A striking observation is that many genes with unknown function are both up- and downregulated uniquely by KAc and CCCP (Fig. S2 in Appendix C). T-profiler also revealed the significant induction of the SubtiList gene groups ‘5.1 from B. subtilis’ and ‘6 no similarity’ (Table S4 in Appendix C).

4.4.11. Testing combinations of stress may reveal the significance of the involved transcriptional responses

We tested different combinations of stresses to see whether certain interactions will cause an increased or reduced growth phenotype. These results may give an insight on the significance of certain transcriptional responses. The combined effects of two compounds can be synergistic, antagonistic, or additive. The exact definitions and the design of experiments that can reveal such interactions are not entirely clear and debatable (Greco et al., 1995, Odds, 2003). However, differences or similarities observed in the transcriptional profiles of stresses may explain why the combination of these stresses cause an increased or reduced sensitivity. For example, when a stress induces a response that is essential for survival and another stress represses the same response also being essential, the combination of stresses will likely result in a synergistic effect. Next, when two compounds induce similar vital responses at a similar time, the combined effects will likely be additive. (Comparable to the addition of twice the amount of the same stress.) Stresses that induce unrelated and/or non-essential overlapping responses may lead to an additive combined effect. Finally, when
similar responses are induced by two different stresses at a different time after stress administration, the combined effects may be antagonistic. (Stress A already induced the response that is required for the tolerance to stress B.) Many other examples can be given that may lead to synergistic, antagonistic, or additive effects.

Previously we have shown that KS renders the cells resistant to cerulenin (CL) (Ter Beek et al., 2008). Here we also tested the simultaneous addition of CL and KAc or CCCP (Table 4.3). Interestingly, the simultaneous addition of CL and KAc also resulted in an antagonistic effect, although less pronounced than the combination of CL and KS (Fig S7A in Appendix C). The combination of CCCP and CL seem to result in an additive effect. The latter can be explained by the absence of a FapR response in CCCP-treated cells. The other data is more difficult to interpret, also since the details of the transcriptional response to CL (besides the clear induction of the FapR regulon after 45 min) are not provided (Schujman et al., 2003). The less pronounced antagonistic effect of KAc and CL, as compared to KS and CL, may be due to the downregulation of gene expression of fabHA, fabHB and fabF, the genes encoding targets of CL. Consequently, the induction of the FapR regulon normally seen in cells treated with CL is less or absent. The fast induction (maximally at 10 min) of the FapR regulon in KS-treated cells may be sufficient to overcome the effects of CL. Although no simple explanation can be given on the basis of these results, they do further corroborate the involvement of the FapR regulon in KS- and KAc-treated cells. However, the possible interactions of potassium (or the anions) with CL should not be ruled out.

We also tested different combinations of weak organic acids (Table 4.3). All different combinations seemed to give an additive effect. This may indicate that the distinct responses seen in these stresses are likely not essential and/or the essential responses do overlap.

Finally, we tested the effect of salt stress in combination with the weak organic acids or CL. Hyperosmotic shock caused by NaCl was used for several reasons. Osmotic shock induces specific responses (e.g. the opu genes, discussed above), the GSR, the Fur regulon, and is reported to cause changes in the lipid and cell wall composition (Beales, 2004, Hecker et al., 2007, Lopez et al., 2006, Lopez et al., 1998, Wood et al., 2001). Additionally, hyperosmotic shock is suggested to increase the membrane permeability for protons (Vindelov & Arneborg, 2002). Salt combined with either CL, KS or KAc seemed to result in

<table>
<thead>
<tr>
<th>GI %</th>
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<th>KS</th>
<th>KAc</th>
<th>CCCP</th>
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<td>28.5 (1.2)</td>
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<tr>
<td>KAc</td>
<td>35.6 (2.3)</td>
<td>40.3 (2.4)</td>
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<tr>
<td>CCCP</td>
<td>27.9 (1.1)</td>
<td>43.4 (0.6)</td>
<td>50.4 (1.2)</td>
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<tr>
<td>CL</td>
<td>71.0 (1.9)</td>
<td>61.9 (1.7)</td>
<td>66.8 (0.8)</td>
<td>77.4 (1.9)</td>
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<tr>
<td>NaCl</td>
<td>22.2 (2.9)</td>
<td>45.4 (1.8)</td>
<td>42.6 (0.4)</td>
<td>113.4 (4.3)</td>
<td>82.0 (0.4)</td>
</tr>
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</table>

*The following end-concentrations of stress were used: 3 mM KS, 25 mM KAc; 0.85 µM CCCP, 5 µg/ml CL, and/or 0.5 M NaCl. The values represent the average of two biologically independent experiments, each consisting of minimally three technical replicates. The standard deviation is shown in brackets.*
additive effects. Strikingly, the combined addition of CCCP and NaCl showed a clear synergistic effect. The opposite regulation of the Fur response upon CCCP and NaCl stress may provide one explanation for the observed growth effect. Cells stressed with NaCl do actually experience an iron limitation (Hoffmann et al., 2002). Therefore, the downregulation of the Fur regulon in CCCP-treated cells may interfere with this. The induction of the Fur response seen in KS and KAc treated cells may be non-essential and therefore no synergy is seen in the combination of CCCP and KS or KAc. Furthermore it has been shown that the desaturation of fatty acids in the membrane by des in cyanobacterium Synechocystis sp. PCC 6803 increases the tolerance of photosynthetic machinery to salt stress (Sakamoto & Murata, 2002). The stronger downregulation of des by CCCP than by KAc might increase the sensitivity to salt stress. A study performed by Lambert and Bidlas (2007) with Aeromonas hydrophila illustrated that combinations of NaCl, low pH, acetate, sorbate, propionate and benzoate, did not give synergistic effects. CCCP was unfortunately not tested. An extended matrix of specific stresses with known targets and responses should be tested to elicit the significant responses caused by weak organic acids.

4.4.12. Concluding Remarks

The growth inhibition curves determined at different pH values revealed the similarities and differences of the growth-inhibitory effects caused by the three tested weak organic acids. As expected, the undissociated form of the acids dominated the inhibitory effect (Fig. 4.1). The effectiveness of the acids correlated to their solubility in the membrane. The anions also contributed to the reduction in growth. The relative contribution to the growth inhibition of CCCP was slightly more than that of Ac− and far more than that of S− (Fig. 4.2). The delocalization of the negative charge over the anion likely lowers the energy-barrier to enter the membrane. Conversely, HS contributed most to the growth inhibition compared to the undissociated forms of the other two acids used in this study.

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. 4.10. 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 and the induction of the stringent response. However, the timeframe of the conducted experiment did not reveal an induction of sporulation. 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 by the influx of protons. In addition, weak organic acids may interfere directly with the nutrient uptake machinery or enzymes further downstream the metabolic pathway. Interestingly, weak organic acids induced distinct responses that all indicate adaptations of the cell envelope.
Fig. 4.10. 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.

downregulation of desaturase des in CCCP- and KAc-treated cells indicates a possible decrease in the fluidity of the membrane. 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. 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). 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. Although some responses involved in the adaptation to changes in osmolarity were observed (changed expression of ktrAB and opuCA-CD in KAc and CCCP-treated cells), there are no clear clues whether the internal anion concentrations led to a significant rise in osmolarity. Interestingly, we found the opposite response of Fur-mediated genes seen in weak organic acid-stressed cultures. KS and KAc both derepressed the Fur regulon; however, CCCP caused a clear downregulation of the genes negatively regulated by Fur. Acetate-stressed cells clearly seem to shift their metabolism to the utilization of acetate by the upregulation of alsSD and acsA, and the downregulation of pdhABCD. 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.

In conclusion, based on the transcriptional responses observed, the main effects of weak organic acids are uncoupling of the proton gradient and possible interference with the cell envelope. The absence of the general stress and SigM-mediated response in sorbic acid-treated cells indicate that acidification of the cytosol contributes less to growth inhibition than is the case of CCCP- or KAC-stressed cells. Since the growth inhibition caused by the compounds was similar (~30%), sorbic acid stress seems to be more focused on the cell
membrane. The synergistic effects seen between salt and CCCP and not between salt and KS or KAc, also exemplified the differences between the modes of action of these compounds. Additional functional experiments (e.g. testing specific mutants) should be performed to elicit the responses significant in the tolerance to weak organic acids. This could be done by testing specific mutants, or increasing the matrix of stress combinations with specific compounds that interfere with known cellular targets and responses.

4.5. Acknowledgements

We would like to express our gratitude to Floyd Witting of the Microarray Department of Amsterdam for coordinating the hybridization and scanning of the microarray slides. We acknowledge Alicia Prats Tur for her technical assistance. Gertien J. Smits is thanked for helpful discussions.