Weak organic acid stress in Bacillus subtilis

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General Discussion
7.1. Effects of weak organic acid stress in *Bacillus subtilis*

The main goal of this thesis was to reveal and understand the responses of *Bacillus subtilis* to weak organic acid stress and the possible resistance mechanisms involved. At the end of the General Introduction (Chapter 1) the following basis question was raised: "What does *B. subtilis* do to battle against stress caused by weak organic acids?". We are now able to answer this question to a significant extent. Although the weak organic acids tested in this research exhibited a generic transcriptional response, each compound also clearly induced distinct reactions.

7.1.1. Uncoupling, energy stress, and acidification

The uncoupling effect caused by the entry of protons into the cytosol and the possible resulting acidification is thought to be one of the main modes of action of weak organic acids (Beales, 2004, Brul & Coote, 1999, Davidson, 2001, Piper et al., 2001). It is generally agreed that the efficiency of energy conservation is affected by the above events (see e.g. Holyoak et al., 1999). Transcriptome analysis of vegetative *B. subtilis* cells stressed with sorbic acid, acetic acid and carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) revealed responses that are normally seen upon nutrient limitation (Ter Beek et al., 2008; Chapters 2 and 4). This nutrient limitation response was exemplified by the derepression of the carbon catabolite control (CcpA-mediated) observed in weak organic acid-stressed cells and the induction of the RelA-mediated stringent response (Fig. 7.1). Thus, the generic transcriptional response is in agreement with the inferred reduction in the proton gradient and associated cellular physiological events.

By using a pH-sensitive GFP derivative (pHluorin) we are now able to monitor the intracellular pH (pH$_i$) of *B. subtilis* cells *in vivo* (Chapter 5). Potassium sorbate (KS), potassium acetate (KAc), potassium benzoate (KB), and CCCP all lowered the pH$_i$ of *B. subtilis* (Fig. 7.1). However, when the growth inhibition was taken into account, CCCP was clearly most efficient in lowering the pH$_i$. Responses that are known to be induced upon low pH stress in *B. subtilis* are the SigB-mediated general stress response (GSR) and the SigM-mediated response (Hecker & Volker, 2001, Thackray & Moir, 2003). The GSR is also induced by energy stress and is preceded by a drop in ATP (Zhang & Haldenwang, 2005). Both responses were clearly induced in CCCP-treated cells. Although ~ 30% of growth inhibition caused by KS, KAc, and CCCP accompanied a drop in pH$_i$ (Chapter 5), the SigM response was not observed in cultures stressed with KS or KAc (Chapter 4). In addition, the GSR was not induced in cells challenged with sorbic acid (Ter Beek et al., 2008; Chapter 2). This might indicate that both sigma factor-mediated responses need a threshold drop in pH to be switched on, or acidification is not a direct signal for the induction of the SigB and SigM responses. A titration with weak organic acids, while monitoring the drop in pH$_i$, the energy-status of the cell (ATP/ADP ratio), and the induction of both responses in the wild-type and *sigB* and *sigM* mutant strains, will give further clues on the significance and inducing signals.
of the GSR and SigM responses. In addition, anchoring pHluorin to the membrane by coupling it to a membrane protein will allow for the more direct monitoring of the (local) proton gradient behaviour as opposed to the behaviour of the ions present in the bulk of the cytosol measured with the current approach.

Fig. 7.1. Overview of the effects of weak organic acids observed in B. subtilis. Transcriptional responses are shown by blue arrows. The red arrow indicates gene products important in weak organic acid tolerance. Acidification of the intracellular pH (pH) was observed using a pH-sensitive GFP derivative (pHluorin) (green arrow). The letters S, A, C, and B indicate the effect was observed in cells stressed with sorbic acid, acetic acid, CCCP, and benzoic acid, respectively. See text for details.
7.1.2. Anion accumulation?

Although the anion can accumulate to very high concentrations in the cell, we did not discover clear responses that indicate an increase in intracellular osmolarity. Surprisingly, KAc induced the opuCA-CD genes and repressed ktrAB, while CCCP-stressed cells showed the opposite profiles. KtrAB is a high affinity K⁺ uptake system and OpuCA-CD a high affinity uptake system for compatible solutes (Fig. 7.1 and discussed in Chapter 4).

7.1.3. Membrane disruption and adaptation

Depending on their hydrophobicity, weak organic acids are thought to disrupt the membrane (Hirshfield et al., 2003, Stratford & Anslow, 1998). More lipophilic acids (higher log Kow values) will have a higher preference for the cellular membrane compared to more hydrophilic ones. We obtained various results from which we inferred that the integrity of the membrane may be affected by the preservative, upon which the cell then responds with reactions to maintain (membrane) homeostasis (Fig. 7.1). The transcriptional studies clearly revealed changes in the expression profiles of genes encoding proteins involved in membrane biosynthesis and adaptation (Ter Beek et al., 2008; Chapters 2 and 4). Remarkably, such responses were weak organic acid specific. For instance, the FapR regulon was induced in KS-stressed cells. However, KAc-treated cells repressed these biosynthesis genes and CCCP did not alter the expression of the regulon. The gene encoding desaturase (des) was repressed in KAc and CCCP-challenged cells. Breakdown of branched amino acids used for the synthesis of branched lipids was observed only in KS-stressed cultures. Repression of regulons involved in the maintenance of the cell envelope and cell wall (SigX, SigW, and YvrH) was detected using the weak acid compounds (Fig. 7.1). The plasma membrane and cell wall are tightly connected and therefore, an alteration in one of the two will likely affect the other component.

Interestingly, a screen for sorbic acid-susceptible genes with a transposon mutagenesis library identified two genes possibly involved in membrane biosynthesis (Chapter 3). Inactivation of a gene encoding a FabG homologue, ymfI, showed a sensitive phenotype for weak organic acid stress. Remarkably, a mutant strain with a transposon inserted in the unknown ymfM gene revealed hypersensitivity towards KS and KAc stress, but not to CCCP stress. A conditional mutant of the downstream gene in the genome, pgsA, showed a similar phenotype as the ymfM transposon mutant on solid plates. The pgsA gene codes for the essential phosphatidylglycerol synthase involved in lipid biosynthesis. Since the sequence of YmfM shows a possible DNA binding domain, we speculated that YmfM might be a transcriptional regulator of pgsA. However, very recent studies in Escherichia coli and Caulobacter crescentus, identified a new player in bacterial (rod-shape) cell morphogenesis: RodZ, a YmfM homologue (Alyahya et al., 2009, Bendezu et al., 2009, Shiomi et al., 2008). Although a B. subtilis knock-out mutant of ymfM could not be created, Alyahya et al. (2009) generated a YmfM overproducing strain showing similar cell shape defects as the C.
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crescentus RodZ overexpressor strain. The helix-turn-helix motif in RodZ is suggested to interact with the actin homologue MreB (Bendezu et al., 2009). MreB, essential for the elongation phase between each cell division, forms helical structures that are thought to direct helical insertion of new peptidoclycan cell wall along the cell circumference (Jones et al., 2001, Kruse et al., 2003).

Whether YmfM (RodZ) interacts with DNA or MreB and whether the protein is more involved in plasma membrane than in cell shape (cell wall) homeostasis remains to be elucidated. Our preliminary results show that ymfM resides in the same operon as pgsA and the ymfM transposon mutant displays modifications in membrane composition (data not shown). However, as mentioned above, the plasma membrane and cell wall are closely linked and therefore, an adjustment in one of the two will likely affect the other cell component. Future studies at our laboratory will focus on YmfM as a possible regulator, as well as cell and colony morphology of the ymfM transposon mutant and YmfM overexpressor strain when grown under environmental and energy stress conditions. We will assess the localization of YmfM by testing a GFP-YmfM fusion, and verify whether overexpression of YmfM, PgsA, and Ymfl reduces the susceptibility to weak organic acid stress. Although ymfM and ymfI are expressed during spore germination and outgrowth, their roles in these processes remain unclear (Keijser et al., 2007; discussed in Chapter 3) Therefore, the germination and outgrowth efficiency of these mutants should also be tested, as well as the resistance properties of their derived spores.

7.2. Use of the growth medium: rich & undefined vs. minimal & defined

In this thesis two types of media were used to culture the cells: a defined minimal medium and undefined rich media. Most experiments were performed in the defined medium, which was heavily buffered with MOPS and contained glucose, glutamate and ammonia as the carbon and nitrogen sources (see Chapters 2, 4, 5 and 6). By using this established Bacillus medium (see e.g. Hu et al., 1999, Wray & Fisher, 2007) the unwanted presence of weak organic acids, as is often the case in undefined rich media such as LB broth, was avoided. However, for the identification of novel weak organic acid-susceptible genes using the transposon mutagenesis library (Chapter 3), we did culture and stress the B. subtilis cells in LB. Transposon mutagenesis was used to create a random mutant library that was subsequently screened for sorbic acid susceptible mutants. A rich medium was used so that as many as possible mutants were able to grow with relatively normal rate prior to the screen (see Chapter 3 for further details).

We are now well aware that that under different conditions (change from rich to minimal medium) other genes might become important for weak-acid adaptation and survival. For instance, B. subtilis cells grown in defined medium induced the yhcA gene when stressed with KS (Ter Beek et al., 2008; Chapter 2). Inactivation of yhcA revealed a resistant phenotype. In rich medium however, the yhcA mutant showed a sensitive phenotype (our unpublished
results). The genes inactivated in the mutants showing an altered weak organic acid susceptibility (Chapter 3), did not show changed expression upon KS, KAc or CCCP stress (Chapters 2 and 4). Although other reasons may explain this observation (further discussed below) the different use of the growth medium may be one of them.

From the examples mentioned above it is clear that the growth medium partly determines (steers) the outcome of the experiments. This aspect is often neglected in current studies. Therefore, in future research it is important to define the growth conditions carefully. Comparison of the effect of the use of different media is time consuming, however it may reveal additional genes important in stress tolerance.

7.3. Low correlation between regulation of genes and phenotype of mutants: transcriptional responses vs. fitness

In chapter 2 we analysed the transcriptional response of sorbic acid-stressed B. subtilis cells. A number of (strongly) induced and repressed genes with an interesting possible function were selected to investigate further by testing specific mutants for their susceptibility to KS stress. Remarkably, the inactivation of the selected genes did not reveal sensitive phenotypes (Ter Beek et al., 2008; Chapter 2). For instance, although the ureA gene was strongly induced by KS, a urease mutant showed a similar phenotype to the WT when stressed with KS. In addition, no sensitive phenotypes were discovered when the KS induced genes yhcA, padC, yxkJ and fabHB were inactivated and stressed with KS. It seems there is a low correlation between the regulation of genes and the phenotype of mutants. Previous studies in S. cerevisiae reported that inactivation of responsive genes often does not lead to a phenotype (see e.g. Giaever et al., 2002). A mutant is already adapted prior to the stress applied and might therefore show different responses upon stress. In addition, the mRNA levels do not necessarily represent the amount of proteins synthesized. However, since KS-treated cells induced 256 genes significantly (Ter Beek et al., 2008; Chapter 2), it might very well be that inactivation of some of these genes will lead to a sensitive phenotype and that the ‘key’ regulated genes were not yet found.

By performing comparative transcriptomics a generic transcriptional response was discovered (Chapter 4). Inactivation of genes representing these generic responses might reveal essential resistance mechanisms. On the other hand, the specific responses observed in cells stressed with KS, KAc and CCCP can give clues on compound-specific resistance mechanisms. In our view, interesting candidates of genes to further investigate are: KAc and CCCP-regulated des (desaturase), and CCCP-regulated sodA (superoxide dismutase). Additionally, the importance of CcpA, Fur, and the RelA-mediated stringent response (regulated by all three compounds) should be investigated using mutants of ccpA, fur and relA, respectively. Finally, the effects of the inability to elicit a given sigma-factor response should be studied in more detail. In particular, these concern: SigB (induced by KAc and
CCCP), SigM (induced by CCCP), SigW (repressed by KS and CCCP), and SigX (repressed by KS and KAc).

Remarkably, none of the sorbic acid-susceptible genes found in the transposon mutagenesis screen were differentially expressed in KS-treated cells. Only the ytrA gene, which was downregulated by solely CCCP, was identified in the transposon mutagenesis screen as a sorbic acid-susceptible gene. However, the transposon mutant showed a resistant phenotype on plate for KS stress and was sensitive in liquid medium to CCCP stress. It will also be of importance to verify effects of mutations in strains on their physiology upon culturing the cells in either well defined minimal growth media or undefined rich media (see the discussion above).

7.4. Impact of heterogeneity: population vs. single-cell studies

Most research in this dissertation was done with whole populations (Chapters 2 – 5). However, research has shown that *B. subtilis* responds heterogeneously to stress conditions (reviewed in Dubnau & Losick, 2006, Lopez *et al*., 2009, Smits *et al*., 2006, Veening *et al*., 2008b). We also showed in Chapter 6 that the general stress response and sporulation are not induced in the same cells at the same time when encountering glucose depletion (Hornstra *et al*., 2009, in press; see Chapter 6). Also the expression of pHluorin showed a heterogeneous response (Chapter 5). The responses observed in weak organic acid-stressed *B. subtilis* cells might also have a heterogeneous character. The transcriptional responses analysed in Chapters 2 and 4 resemble the average responses of billions of individual cells. When sub-populations in a culture stressed with weak organic acids respond differently the strength of the responses are levelled out and even may found not to be significant anymore after microarray data analysis. As discussed in Chapter 6, a heterogeneous response may have significant impact on the survival rate of the stressed cells by for instance influencing spore resistance properties (Hornstra *et al*., 2009, in press; see Chapter 6).

Therefore, future studies on weak organic acid stress should not only focus on whole population studies. Single-cell experiments are needed to reveal to which extent of heterogeneity the responses to weak organic acids are. This type of research is especially important for the food industry, since small amounts of surviving cells or spores may significantly influence the chances of food spoilage or toxin occurrence in products. An important viability parameter such as the pH is in this context ideally studied on the single-cell level. For this, the behaviour of the pH-sensitive GFP derivative, pHluorin (Chapter 5) is investigated with fluorescence microscopy and flow cytometry. In addition, monitoring the induction of specific stress responses, like the GSR and sporulation, with fluorescent reporters (Hornstra *et al*., 2009, in press; see Chapter 6), performing fluorescence assisted cell sorting (FACS), and determining the resistance properties of subsequently formed spores will give further clues on the origin of variability in stress resistance of spores.
7.5. A final word

In conclusion, it is clear that the study of the molecular basis of weak organic acid-stress response is at a crossroad. The molecular tools have developed such that crucial details of cellular building blocks involved in weak organic acid-stress response have been identified in *B. subtilis*. The challenge is now to link the gained insight in molecular details to the physiological observations. The *in situ* measurement tools for pH, as well as the reporter proteins for the GSR, and specific stress responses are first examples of developments that bring this goal nearer.