Weak organic acid stress in Bacillus subtilis

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General Introduction
1.1. *Bacillus subtilis*, a spore-forming bacterium of relevance to the food industry

Ingredients of many food products are contaminated with spore-forming bacteria. The food industry is often confronted with this problem, especially in the manufacturing of soups, sauces, and similar savoury products, as well as of dairy products (see e.g. Oomes *et al.*, 2007, Scheldeman *et al.*, 2006). The ability of bacteria to form highly-resistant endospores that can withstand extreme temperatures makes them difficult to control. One extreme, but relevant example are the spores from *Bacillus sporothermodurans* strain IC4 that are able to survive 3 min of 131°C of wet-heat (Kort *et al.*, 2005). In fact, complete inactivation of bacterial spores is often impossible without altering the quality of the food. In addition, food-processing techniques may even give spore-formers an advantage over other microorganisms, since sub-lethal heat-treatments may promote (activate) germination whilst competing species will have been killed during the thermal preservation step (Klijn *et al.*, 1997).

Bacterial spores identified in food products originate from species of the genus *Clostridium* and *Bacillus*. Clostridia are obligate anaerobes that can cause severe food poisoning. For example, vegetative cells of *Clostridium botulinum* can produce the neurotoxin botulinum, the most toxic protein known (Montecucco & Molgo, 2005, Peck, 2006). Another well known species of this genus is *Clostridium perfringens* that can cause food-borne gastrointestinal diseases in humans and animals (Miki *et al.*, 2008, Rahmati & Labbe, 2008). Compared to Clostridia, the spores of Bacilli are equally, or even significantly more, heat resistant (Oomes *et al.*, 2007, van Asselt & Zwietering, 2006). *Bacillus anthracis* is the most notorious species of this genus. Although not commonly found in food, *B. anthracis* is responsible for the production of exotoxins, which cause anthrax in humans and animals (Dixon *et al.*, 1999). *Bacillus cereus*, often found in dairy products can cause both food spoilage and more importantly, food poisoning (Huis in ’t Veld, 1996). For example, between 1993 and 1998 *B. cereus* accounted for 12% of the food-borne disease outbreaks in the Netherlands (Stenfors Arnesen *et al.*, 2008). Although *Bacillus subtilis* rarely causes food poisoning, it is a recurrent source of food spoilage and therefore responsible for economical losses. For instance, *B. subtilis* is accountable for rope formation on spoiled bread (Pepe *et al.*, 2003).

*B. subtilis* is commonly found in soil and was first discovered by Christian Gottfried Ehrenberg in 1835 who named it *Vibrio subtilis* (Ehrenberg, 1835). Almost thirty years later, Ferdinand Cohn gave it its present name, which means thin rod (Cohn, 1872). As discussed above, this Gram-positive bacterium is able to form highly-resistant endospores (Fig. 1.1). When *B. subtilis* strain 168 was generated and identified to be highly competent for natural transformation more than fifty years ago, it became significantly easier to genetically manipulate this organism (Sonenshein *et al.*, 2002). Consequently, *B. subtilis* is now by far the most studied and best characterized Gram-positive bacterium and became its representative as a model organism. The publishing of its complete genome sequence in
1997 opened the door for genome-wide studies, using *e.g.* microarray technology or proteomics (Kunst *et al.*, 1997).

### 1.2. Transcriptional programs and adaptation mechanisms of *B. subtilis* in response to stress and starvation

Microorganisms need to sense and adapt to changes (stress) in the environment in order to survive. Basically, any change from ideal conditions can be considered as stress. However, it is not always easy to define what exactly a 'stressful' or 'ideal' environment is. For example, salt-stressed *B. subtilis* cells grow slower than non-stressed control cells. Conversely, the cells adapted to the salt stress are far more resistant to a future heat-shock than cells that were not challenged by the first stress-factor salt (Volker *et al.*, 1999). Here we define stress as a factor causing a reduction in the growth rate and/or a decrease in the viability of the organism in comparison to a non-stressed control within the time-frame of the experiment. Thus, this definition also applies to (nutrient) starvation, yet, for clarity we use both terms. The former condition is in literature often referred to as 'environmental stress', whereas the latter is in many papers referred to as a prime example of 'energy stress'.

When nutrients are depleted or conditions are far from optimal, *B. subtilis* has highly developed adaptation mechanisms that can be switched on in order to survive (Table 1.1). The most generic response to various kinds of stresses is called the general stress response. The cells induce this SigB-mediated response not only to directly cope with the stress, but also to provide cross-protection (discussed in more detail below). Under nutrient limiting and high cell density conditions *B. subtilis* is able to form highly resistant endospores. This process, called sporulation, is controlled by master regulator Spo0A (discussed in more detail below). The spores will germinate and grow out to vegetative cells when conditions are not limiting anymore.

![Fig. 1.1. Phase-contrast microscope picture of *B. subtilis* cells forming endospores (bright spot inside the cell). A released (mature) spore is shown on the right.](image-url)
Table 1.1. Overview of relevant (generic) responses of *B. subtilis* induced by stress and/or limitations.

<table>
<thead>
<tr>
<th>(Generic) response</th>
<th>Key regulator</th>
<th>Induced by</th>
<th>Reviewed in e.g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>General stress response</td>
<td>SigB</td>
<td>energy stress (<em>e.g.</em> glucose-, oxygen-, phosphate-starvation, CCCP, azide) sudden environmental stress (<em>e.g.</em> NaCl, EtOH, low pH, heat, blue light) growth at low temp.</td>
<td>Hecker et al. (2007)</td>
</tr>
<tr>
<td>Sporulation</td>
<td>Spo0A</td>
<td>nutrient starvation &amp; high cell densities</td>
<td>Piggot and Hilbert (2004)</td>
</tr>
<tr>
<td>Competence</td>
<td>ComK</td>
<td>nutrient starvation &amp; high cell densities</td>
<td>Hamoen et al. (2003)</td>
</tr>
<tr>
<td>Stringent response</td>
<td>RelA</td>
<td>nutrient starvation (<em>e.g.</em> amino acid-, glucose-, oxygen-starvation)</td>
<td>Ochi (2007)</td>
</tr>
<tr>
<td>Biofilm formation</td>
<td>SinR</td>
<td>nutrient starvation</td>
<td>Lemon et al. (2008)</td>
</tr>
</tbody>
</table>

*B. subtilis* can develop competence for genetic transformation (reviewed in *e.g.* Hamoen et al., 2003). Competent cells are able to take up exogenous DNA and can incorporate this into their own genome. The development of competence is induced by nutrient starvation and high cell densities. The induction of competence starts with sensing the excreted pheromones ComX and competence-stimulating factor (CSF). Via a complex regulatory network the main regulator ComK is then activated and switches on the genes necessary for the development of competence (Hamoen et al., 2003).

The stringent response is induced under non-growing (nutrient limiting) conditions in order to prevent the waste of nutrients by switching off the synthesis of ribosomes and other growth related processes, and inducing adaptive responses to nutrient starvation (Ochi, 2007). Most studies on the stringent response have been performed with auxotrophic strains that highly induce this response under amino acid limiting conditions. The stringent response is mediated by RelA, which synthesizes the alarmone (p)ppGpp in response to the binding of uncharged tRNA to the ribosome. As a consequence, GTP levels drop, influencing transcription, replication and translation.

Dense multicellular communities, called biofilms can be formed under nutrient limiting conditions (Lemon et al., 2008). In biofilms the cells are held together by a matrix of polymers and may appear at the liquid-air interface or on solid surfaces. Cells and spores inside the biofilm are better shielded from the environment and therefore show more resistant properties. The initiation of biofilms is regulated by SinR, the key repressor of genes essential for biofilm formation (Kearns et al., 2005). Activated SinI, through low levels of phosphorylated Spo0A, inhibits the repressing function of SinR.

Besides the responses shortly discussed, *B. subtilis* also has other generic responses and numerous specific responses induced by specific stresses. These responses will be discussed throughout this thesis when appropriate. Here we will discuss in more detail the two most relevant responses of *B. subtilis* towards changes in the environment: the general stress response and sporulation.
1.2.1. The SigB-mediated general stress response

*B. subtilis* cells respond to a variety of stress and starvation conditions by a strong induction of the general stress response (GSR), which is controlled by the transcription factor SigB (Hecker *et al.*, 2007, Hecker & Volker, 2001). One of the largest known regulons, the regulon of master regulator SigB, is composed of more than 150 genes coding for general stress proteins (GSPs) (Petersohn *et al.*, 2001, Price *et al.*, 2001). The majority of SigB-dependent GSPs have as yet unknown function. The main role of SigB-controlled GSPs is to equip the stressed or starved *B. subtilis* cells with nonspecific, multiple and cross-protective stress resistance (Hecker *et al.*, 2007, Hecker & Volker, 2001). For instance, it has been shown that starved wild-type *B. subtilis* cells develop increased resistance to salt stress (Volker *et al.*, 1999).

The expression of SigB-mediated GSR is tightly controlled. The GSR is inactive in exponentially growing cells, while its activation is fast and powerful upon exposure to various environmental and nutritional changes (e.g. both high and low temperature, glucose, oxygen and phosphate starvation) and different chemical stresses (exposure to salt, ethanol, or antibiotics causing cell wall stress such as bacitracin or vancomycin; to mention a few) (Hecker *et al.*, 2007).

The regulation of the GSR is achieved by controlling the activity of its master regulator SigB (Fig. 1.2). SigB is tightly controlled by an intricate signalling cascade, where interactions among SigB, the antisigma factor RsbW (*Regulator of sigma-b W*) and the antagonist RsbV occur via reversible phosphorylations (Price, 2002). In exponentially growing cells RsbW plays a crucial role in silencing of the SigB-regulon by forming a stable complex with SigB, which in turn prohibits SigB from binding to the core enzyme of RNA polymerase (Benson & Haldenwang, 1993). Upon exposure to stress, the release of SigB from the RsbW-SigB complex is caused by the nonphosphorylated antagonist RsbV. Interestingly, the antisigma factor RsbW is also responsible for phosphorylation of the antagonist RsbV, thus ensuring the stability of the inactive complex in growing cells (Dufour & Haldenwang, 1994). The dephosphorylation of RsbV in stressed cells is accomplished by activation of two phosphatases, RsbU and RsbP (discussed below). Dephosphorylated RsbV binds RsbW, which then releases SigB.

Three signalling pathways lead to activation of SigB (Fig. 1.2). These pathways are triggered via three different inputs: energy stress, environmental stress or growth at low temperature (Brigulla *et al.*, 2003, Voelker *et al.*, 1995). Here, we discuss the pathways activated by energy and environmental stress. The pathway leading to activation of SigB regulon caused by growth at low temperature is, until now, very poorly understood.

In *B. subtilis*, energy stress or energy depletion is caused by glucose, phosphate or oxygen starvation as well as exposure to azide, nitric oxide (NO), carbonyl cyanide-m-chlorophenyl hydrazone (CCCP), decoyinine or mycophenolic acid (Hecker *et al.*, 2007).
These energy stress conditions lead to a decrease in the available ATP pool. ATP depletion is thought to be sensed by proteins RsbQ and RsbP (Zhang & Haldenwang, 2005). The protein RsbQ is suggested to activate the phosphatase RsbP by providing its essential cofactor (Kaneko et al., 2005). RsbP in its active form dephosphorylates RsbV, which leads to release of SigB from the inactive RsbW-SigB complex and subsequent induction of SigB-regulon (Vijay et al., 2000). Additionally, the decreased availability of ATP may block or decrease the phosphorylating activity of RsbW that might result in accumulation of nonphosphorylated RsbV (Alper et al., 1996, Voelker et al., 1995). The second proposed mechanism via RsbW appears to play only a small part in induction of SigB-regulon as the rsbP mutant does not show any activation upon energy stress (Hecker et al., 2007). Interestingly, a relA mutant, involved in the stringent response (discussed below) is also unable to activate the energy stress pathway (Zhang & Haldenwang, 2003).

The known environmental stress stimuli of the B. subtilis SigB-regulon comprise exposure to heat or blue light, and treatment with salt, ethanol or acid (low pH) (Hecker et al., 2007). The signalling of environmental stresses is relayed to phosphatase RsbU via a complicated yet flexible multicomponent protein complex named the stressosome (Delumeau et al., 2006) (Fig. 1.2). Recently the molecular architecture of stressosome has been resolved (Marles-
Wright et al., 2008). In exponentially growing cells the stressosome consists of the antagonist RsbS and its paralogs (RsbRA-RsbRD), which shield the protein kinase RsbT, thus preventing it from activating the RsbU phosphatase (Kim et al., 2004, Reeves & Haldenwang, 2007). Upon exposure to environmental stress, RsbT becomes highly active and phosphorylates its antagonist RsbS, which in turn is inactivated and leads to release of RsbT from the stressosome. The released RsbT activates the RsbU phosphatase, which results in induction of SigB, as discussed above. Blue light is sensed by YtvA, another RsbR paralog and associated with the stressosome (Avila-Perez et al., 2006, Gaidenko et al., 2006). Additionally, the involvement of the ribosome in sensing environmental stress was shown (Scott & Haldenwang, 1999, Zhang et al., 2001). Strains unable to produce ribosomal protein L11 or GTP-binding protein Obg are unable to activate the GSR by environmental stress. Finally, the environmental stress signalling pathway is switched off by the RsbX phosphatase which counteracts the kinase activity of RsbT and consequently deactivates the stressosome (Price, 2002, Yang et al., 1996).

1.2.2. Sporulation

*B. subtilis* has the ability to form endospores, which are tough, dormant cells able to survive starvation or exposure to harsh environmental conditions such as heat, hydrolytic enzymes, chemical solvents, detergents, mechanical disruption or even UV- and gamma-radiation (Nicholson et al., 2000, Setlow, 2006). The formation of endospores occurs via the process called sporulation.

The process of sporulation commences with a crucial event: the asymmetric division of the mother cell (Fig. 1.3). This step leads to formation of the small prespore compartment separated from the rest of the cell by the asymmetric septum. The following steps of sporulation comprise the engulfment of the prespore by the mother cell, formation of the spore core, cortex and coat, and finally the release of the newly formed spore from the mother cell (Errington, 2003). Endospore structure is very different from that of a vegetative cell. The spore core is equipped with machinery essential for germination and conversion into a vegetative cell under conditions where nutrients are no longer limiting. The core is highly dehydrated and mineralized due to presence of large quantities of dipicolinic acid and divalent cations (mainly Ca^{2+}). The inner membrane surrounding the core has low permeability for a number of chemicals. The cell wall and cortex form a tight peptidoglycan-based layer, surrounded by an outer membrane, which is in turn enveloped in a multilayered protein coat. These characteristic features provide the endospore with its exceptional resistance properties (Nicholson et al., 2000, Setlow, 2006).

Sporulation is controlled by an extremely complicated signalling network with its most important constituent - the key transcriptional regulator Spo0A (Errington, 2003, Piggot & Hilbert, 2004). More than 10% of all *Bacillus subtilis* genes are controlled (directly or
indirectly) by Spo0A (Fawcett et al., 2000). The functional activity of Spo0A is controlled by phosphorylation. Spo0A is activated upon nutrient limitation and in high cell densities through a complex phosphorelay system comprising a number of kinases (KinA, KinB, KinC, KinD and KinE) sensing various stimuli, like energy potential, redox state, and impaired DNA replication or DNA damage (Piggot & Hilbert, 2004) (Fig. 1.4). The transfer of phosphate from the kinases to Spo0A occurs via two mediator proteins, Spo0F and Spo0B (Burbulys et al., 1991). When the phosphorylated form of Spo0A is present in high concentrations, reached within a certain predetermined time frame, it switches on the process of sporulation. This is accomplished by the induction of the transcription of several important sporulation genes, in particular the early sporulation genes spoIIA, spoIIE and spoIIG. The gene spoIIE together with a bacterial homologue of tubulin, FtsZ, participate in the initial sporulation event of septum formation and asymmetric cell division into the small prespore and the large mother cell (Ben-Yehuda & Losick, 2002). The segregation of the chromosome into the prespore requires the activity of SpoIIIE (Wu & Errington, 1994). The transcriptional program activated in the prespore is governed by the crucial sigma factor SigF. The prespore-restricted activity of SigF is ensured by two specific mechanisms. The first mechanism is based on interplay of
SpoIIAB, which binds to SigF, thereby blocking its interaction with RNA polymerase and SpoIIAA, which in non-phosphorylated form frees the SigF from the complex with SpoIIAB. The dephosphorylation of SpoIIAA, required for its activation and subsequent release of SigF, is accomplished by the specific phosphatase activity of SpoIIE (King et al., 1999, Wu et al., 1998). The dynamics of this process are not yet fully understood, however one suggestion is that SpoIIE is delivered into prespore and there successfully competes with kinase activity of SpoIIAB. The second proposed mechanism is related to the instability of SpoIIAB and its chromosomal position – almost opposite the oriC region (Lewis et al., 1996, Pan et al., 2001). Both mechanisms contribute to proper regulation of SigF activity.

The activation of SigF is followed by immediate activation of mother cell-specific sigma factor SigE. The activation of SigE possibly occurs via its proteolytic processing by the
membrane-bound SpoIIGA protease (LaBell et al., 1987). The SigF and SigE-regulated gene expression ensures the proper differentiation of the two cell types.

In the next stage of sporulation, the prespore is engulfed in the mother cell in a phagocytosis-like process. In the first step of engulfment, most likely catalyzed by SpoIIB (Margolis et al., 1993), the cell-wall material in the septum is degraded. Next step involves migration of the septal membranes, and is controlled by SigE-dependent proteins SpoIID, SpoIIM and SpoIIP (Abanes-De Mello et al., 2002). Final step of engulfment, the fusion of septal membranes at the cell apex and formation of a free protoplast-like spore within the mother-cell cytoplasm, requires the protein SpoIIIE.

Following engulfment, the sigma factor SigG becomes active inside the spore and directs the final events of spore development. The final mother cell-restricted sigma factor active during the last phase of sporulation is SigK. Both SigG and SigK are subjected to complex transcriptional and post-transcriptional regulation mechanisms that are discussed elsewhere (Errington, 2003).

Another important positive regulator of sporulation is the sigma factor SigH, which induces the transcription of more than 80 genes by interacting with core RNA polymerase (Britton et al., 2002). The interplay of both Spo0A and SigH-mediated signalling pathways ensures the correct progress of sporulation (Fig. 1.4). There are three main transcriptional regulators that repress the initiation of sporulation: CodY, whose repressor function is activated by intracellular GTP levels; AbrB, a negative regulator of many repressors; and Soj, an inhibitor of sporulation associated with replication status. The discussion of the complex regulatory network involving the aforementioned signalling proteins is beyond the scope of this introduction.

1.2.3. The issue of heterogeneity in the responses of B. subtilis

As discussed above (Table 1.1), various responses (e.g. sporulation, competence, biofilm formation) are induced under the same nutrient limiting conditions. However, neither all cells within one isogenic culture of B. subtilis switch on all these responses at the same time, nor all cells induce only one type of response. In general, these responses seem mutually exclusive, i.e. a part of the populations switches on one type of response, while another part induces a different response (Lopez et al., 2009). The heterogeneous responses in sporulation, competence and biofilm formation have been extensively studied and reviewed over the recent years (Veening et al., 2008b, Dubnau & Losick, 2006, Lopez et al., 2009, Smits et al., 2006). In conclusion, as a consequence of (intrinsic and extrinsic) noise in gene expression and the ‘design’ of the regulatory networks, the key regulators involved (Spo0A and ComK) can exist in two stable states (i.e. a bistable system). The different states of the regulator (low or high phosphorylation-state for Spo0A and low or high expression of comK) will lead to different responses.
As a consequence of heterogeneity, cells within one population will have different resistant properties, making it more difficult for the food industry to predict and control spoilage of microorganisms.

1.3. Weak organic acids

1.3.1. Weak organic acids as preservatives

In the food industry, weak organic acids are the most commonly used chemical preservatives (see e.g. Russell & Gould, 2003). The acid, or its anionic salt is utilized in a variety of foods and beverages and displays a broad range of antimicrobial activities against spoilage bacteria, yeasts, and moulds (Beales, 2004, Brul & Coote, 1999, Davidson, 2001, Piper et al., 2001). The most active among them are: propionic, acetic, sorbic, benzoic, and lactic acid (Table 1.2). Weak organic acids occur naturally in many fruits and vegetables or

Table 1.2. Overview of commonly used weak organic acids and their properties.

<table>
<thead>
<tr>
<th>Weak organic acid</th>
<th>Chemical structure</th>
<th>pKₐ</th>
<th>Log K&lt;sub&gt;ow&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
<th>E-number&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Used in e.g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionic acid</td>
<td>O</td>
<td>4.88</td>
<td>0.33</td>
<td>E270</td>
<td>bakery products, meat and meat products, pizza, cheese</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>O</td>
<td>4.76</td>
<td>-0.17</td>
<td>E260</td>
<td>pickles, chutneys, sauces, butter, margarine, processed cheese, oils</td>
</tr>
<tr>
<td>Sorbic acid</td>
<td>O</td>
<td>4.76</td>
<td>1.33</td>
<td>E200</td>
<td>dairy products, cheese, cakes and bakery products, shellfish, wine, soups</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>O</td>
<td>4.21</td>
<td>1.87</td>
<td>E210</td>
<td>alcoholic beverages, baked goods, cheeses, gum, frozen dairy, soft sweets, cordials</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>O</td>
<td>3.85</td>
<td>-0.72</td>
<td>E280</td>
<td>fresh and cooked meat products, sour milk products, dressings, soft drinks, fresh fruit and vegetables</td>
</tr>
</tbody>
</table>

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

<sup>b</sup> European Union approved food additives. The numbering scheme follows that of the International Numbering System as determined by the Codex Alimentarius committee.
are the product of biological fermentation processes. Sorbic acid (trans-trans-2,4-hexadienoic acid) was first isolated from unripe berries of Rowan (Sorbus aucuparia) by August Wilhelm von Hoffman in 1859. Benzoic acid naturally occurs in e.g. cranberries, plums, prunes and cinnamon. The fermentation of foods (carbohydrates) by bacteria of the genus Acetobacter, Lactobacillus and Propionibacterium is the main source of naturally produced acetic, lactic and propionic acid, respectively.

1.3.2. Presumed modes of action

Weak organic acids are most effective at lower pH. Besides the concentration of the weak organic acid present, its antimicrobial activity depends mainly on three factors: the pH of the environment, the pKa and the log Kow values of the preservative (Table 1.2). In solution, a weak organic acid exists in equilibrium between the dissociated state (A\(^-\)) and undissociated state (HA) (Fig. 1.5). The distribution of the two forms of the acid is determined by its pKa value and the pH of the environment. Consequently, in more acidic environments the equilibrium is shifted to the undissociated form. Charged molecules, like protons and the anion, are unable to pass the lipid membrane. However, the neutral HA is relatively membrane permeable and able to diffuse over the membrane. Inside the cell a new equilibrium is formed between HA and A\(^-\) (Fig. 1.5). Since, most microorganisms exhibit an intracellular pH near neutrality the equilibrium is shifted to the dissociated state of the acid. Consequently, protons are released into the cytosol, lowering the proton gradient. Part of the energy used to build up the proton gradient (for e.g. ATP synthesis) is thereby instantly lost. Depending on the buffering capacity of the cell, the entry of protons can acidify the cytosol. Changes in intracellular pH 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 resulting acidification of the cytosol 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).

Since the entry of HA into the cell is driven by diffusion, this process continues until the concentrations of HA on the inner and outer membrane leaflets are equal. The higher the initial pH difference between the environment (lower pH) and the inside of the cell (higher pH), the higher the intracellular anion concentration can get (Russell & Diez-Gonzalez, 1998). For instance, it has been reported that Escherichia coli cells treated with 8 mM acetic acid at an extracellular pH of 6.0 can accumulate more than 240 mM acetate, which can lead to an osmotic stress (Roe et al., 1998). Furthermore, depending on the preservative used, A\(^-\) may also affect cytosolic enzymes directly, perturbing metabolic pathways (Russell, 1992). It has been suggested that sorbic acid inhibits the enolase enzyme of glycolysis in Saccharomyces cerevisiae (Azukas et al., 1961). In addition, it was reported that sorbic acid can react with
Fig. 1.5. Overview of generic modes of action of weak organic acids (top part) and possible adaptive responses (bottom part). See text for details.
cysteine, forming a thiol adduct and thereby inactivating enzymes, like fumerase and aspartase (York & Vaughn, 1964). Benzoic acid was shown to inhibit a-ketoglutarate, succinate dehydrogenase and trimethylamine-N-oxide reductase in *E. coli* (Bosund, 1962, Kruk & Lee, 1982). Although not clear how, studies of Eklund showed that, the anions of sorbic, benzoic and propionic acid present in the extracellular medium also contribute, but to a lesser extend than HA, to the growth-inhibitory effect in bacteria (Eklund, 1983, Eklund, 1985). In summary, the antimicrobial activity of weak organic acids is not solely due to undissociated acid, but also caused by the $A^-$ through accumulation inside the cell and, depending on the weak organic acid, inhibition of specific cellular enzymes (Fig. 1.5).

When comparing weak organic acids, the growth-inhibitory effect seems to correlate with their membrane solubility, as reflected by the octanol-water partition coefficient (log $K_{ow}$) (Table 1.2). To obtain similar reductions in growth rate, usually lower concentrations of more hydrophobic weak organic acids are needed than of more hydrophilic ones. For instance, this was shown for benzoic, sorbic, acetic and propionic acid in *S. cerevisiae* (Abbott et al., 2007, Piper et al., 1998). In addition, the transcriptional responses of *S. cerevisiae*, grown anaerobically in chemostats showed more similarities between similarly lipophilic compounds (Abbott et al., 2007). The more lipophilic benzoic and sorbic acids induced responses more related to the cell wall, while the responses induced by less lipophilic acetic and propionic acid focused on membrane-associated transport (Abbott et al., 2007). This observed correlation will likely be the consequence of the faster/easier entry of the less hydrophobic weak organic acid into the cell. However, more lipophilic compounds, like sorbic and benzoic acid, may also reside in the membrane. There they may act more as a ‘classical’ uncoupler (by moving up and down the membrane, transporting protons) and interfere with the membrane and consequently, influence the transport of nutrients (Hirshfield et al., 2003, Sheu & Freese, 1972, Stratford & Anslow, 1998).

In conclusion, no single mechanism appears to explain the antimicrobial activity of weak organic acids. Their modes of action are related to: dissipation of the proton gradient (uncoupling), acidification of the cytosol, membrane disruption, osmotic stress by anion accumulation, and metabolic perturbation by inactivating specific enzymes (Fig. 1.5).

### 1.3.3. Adaptive responses to weak organic acid stress

Depending on the preservative used and the challenged organism, various resistance mechanisms have been described to cope with weak organic acids. In general, it is thought that membrane residing H$^+$-ATPases are able 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) (Fig. 1.5). Studies in *S. cerevisiae* showed that the duration of the lag phase induced by weak organic acids correlated to the drop in intracellular pH and the activity of the H$^+$-ATPases (Holyoak et al., 1996, Lambert & Stratford, 1999). To prevent the accumulation and toxic effects of the sorbate anion, *S. cerevisiae* induces a dedicated ATP binding cassette
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(ABC) transporter, Pdr12p, to extrude the anion at the cost of ATP (Holyoak et al., 1999) (Fig. 1.5). The actions of both the H+-ATPases and Pdr12p, however, may reduce energy resources significantly and even cause an energy stress (Bracey et al., 1998, Holyoak et al., 1999, Holyoak et al., 1996). In addition, the expulsion of the proton and the anion may create a futile cycle (Brul & Coote, 1999).

The membrane is one of the first barriers a weak organic acid has to pass. Changing the properties of the membrane might decrease the entry of the acid into the cell. The adaptation of the membrane upon stress by more lipophilic weak organic acids has been reported. For instance, Golden et al. (1994) showed changes in fatty acid composition in sorbate-stressed Zygosaccharomyces rouxii cells. Furthermore, benzoate-adapted S. cerevisiae and Zygosaccharomyces bailii cells reduced their permeabilities to benzoic acid, implying a possible resistance mechanism (Henriques et al., 1997, Warth, 1989). S. cerevisiae possesses an aquaglyceroporin (Fps1) that normally transports water and small molecules. However, upon acetic acid stress, Fps1 is degraded, thereby increasing the tolerance to the weak organic acid.

Another resistance mechanism observed in certain microorganisms is the ability to degrade the acid in order to minimize the effects of the anion (Fig. 1.5). Interestingly, Z. bailii is able to degrade benzoate and sorbate (Mollapour & Piper, 2001), and species of Penicillium can decarboxylate sorbate to 1,3-pentadiene (Kinderlerer & Hatton, 1990). Recently, it was also shown that spoilage yeasts of the Saccharomyces genus and the mold Aspergillus niger are able to decarboxylate sorbate (Plumridge et al., 2008, Stratford et al., 2007). Besides the fact that acetic, propionic, and lactic acid are used as food preservatives, these acids are also fermentation products of certain microorganisms. When the preferred carbon source is depleted many of these microorganisms can change their metabolism to the utilization of the earlier produced fermentation products. Likely, these conditions will increase the tolerance to the weak organic acids.

Compared to yeasts, the weak acid resistance mechanisms in bacteria are largely unknown. Although E. coli challenged with acetic or propionic acid induced the RpoS-mediated GSR, it was not tested whether this response was important for weak organic acid tolerance (Polen et al., 2003). In bacteria low pH stress, caused by e.g. hydrochloric acid and is therefore different than weak organic stress, can induce several systems to counteract a drop in the internal pH. The GSR and the SigM regulon are both induced in B. subtilis upon low pH stress (Hecker & Volker, 2001, 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 *E. coli* increased the resistance to weak organic acids (Baik *et al.*, 1996).

Although *B. subtilis* is a relevant food spoiler, until now the responses and resistance mechanism of this Gram-positive bacterium upon weak organic acid stress are largely unknown. No data is available whether the phospholipid membrane of *B. subtilis* is involved in the adaptation to weak organic acid stress.

### 1.4. The plasma membrane of *Bacillus subtilis*

The plasma membrane is the ultimate barrier between the outside world and the inside of any living organism known on this planet. It prevents the cellular contents from leaking out of the cell and serves as protection against many harmful compounds. The plasma membrane of *B. subtilis* mainly consists out of proteins embedded in a phospholipid bilayer. Phospholipids are composed of fatty acids linked through an ester bond to *sn*-glycerol-3-phosphate (G3P). Different fatty acids, varying in length, saturation and branching, can be used as precursor. In addition, the phosphate headgroups of the lipids are modified in various ways leading to complex constituents with varying properties. Not surprisingly, many enzymes involved in the biosynthesis of the plasma membrane are essential. In the following sections, the composition and synthesis of the plasma membrane are discussed. In addition, we discuss the regulation of the membrane composition when cells are exposed to stressful culture conditions.

#### 1.4.1. Fatty acid composition and biosynthesis

In *B. subtilis* many different types of acyl-lipid chains are found in membranes (de Mendoza *et al.*, 2002, Zhang & Rock, 2008a). Their occurrence highly depends on the specific strain of *B. subtilis* and the conditions it experiences. The majority of fatty acids present in the membrane of *B. subtilis* are branched (either iso or anteiso) and consist of pentadecanoic acids anteiso-C_{15:0} and iso-C_{15:0} (Fig. 1.6). Other fatty acids commonly found in the membrane are anteiso-C_{17:0} and n-C_{16:0}.

*B. subtilis* uses a type II fatty acid synthase system to synthesize fatty acids. This means that distinct monofunctional enzymes are utilized sequentially to generate the necessary fatty acids. The biosynthesis of fatty acids can be divided into two parts: the initiation and the elongation cycle. The first step in the initiation of fatty acid biosynthesis in *B. subtilis* is the conversion of acetyl-CoA to malonyl-CoA by essential acetyl-CoA carboxylase (ACC) (Fig. 1.7A). The essential genes *accA*, *accBC* and *accD* code for this four-subunit-containing complex that requires biotin as a cofactor (Kunst *et al.*, 1997, Marini *et al.*, 1995). Next, the malonyl-CoA is transferred to the holo form of acyl carrier protein (ACP) via essential malonyl transacylase FapD (Morbidoni *et al.*, 1996). The apo form of ACP is encoded by the essential *acpA* gene. Apo-ACP and Coenzyme A (CoA) are converted to the holo form and 3,5-ADP by
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**Fig. 1.6.** Commonly found saturated fatty acids in *B. subtilis*. See text for details.

holo-ACP synthase AcsS, another essential enzyme (Mootz *et al.*, 2001). Holo-ACP binds all further intermediates of the fatty acid biosynthesis. The initiation of both straight- and branched-chain fatty acid biosynthesis is performed by the two isoforms of β-ketoacyl-ACP synthase, FabHA and FabHB (Choi *et al.*, 2000). Although, the *fabHA* and *fabHB* genes are non-essential (Kobayashi *et al.*, 2003), the double mutant is nonviable (Thomaides *et al.*, 2007). In this condensation reaction, malyonyl-ACP is attached to the new acyl chain from various acyl-CoA substrates. The use of acetyl-CoA finally leads to a straight-chain fatty acid. Branched-chain fatty acids are formed when isovaleryl-, isobutyryl-, or 2-methylbutyryl-CoA substrates are used (Kaneda, 1977). These three acyl-CoA forms are produced when
Fig. 1.7. The type II fatty acid synthase system of *B. subtilis*. The biosynthesis of fatty acids can be divided in (A) the initiation and (B) the elongation cycle. Essential enzymes are underlined. See the text for details. Abbreviations: ACC: acetyl-CoA carboxylase; CoA: coenzyme A; ACP: acyl carrier protein.
respectively, leucine, valine, or isoleucine are degraded. The bkd operon, regulated by BkdR, encodes the enzymes responsible for the degradation of these branched-chain amino acids (Debarbouille et al., 1999). The various β-ketoacyl-ACP intermediates produced by FabHA or FabHB are then further converted in the elongation cycle, via essential β-ketoacyl-ACP reductase (FabG), β-hydroxyacyl-ACP dehydrase (YwpB?), and enoyl reductases (FabI or FabL) to an acyl-CoA that is two carbon atoms longer than the original acyl-ACP (de Mendoza et al., 2002, Heath et al., 2000) (Fig. 1.7B). Again, individual inactivation of fabI or fabL is compatible with life, however a double mutation is lethal (Thomaides et al., 2007). Subsequent rounds of elongation are initiated by the essential elongation condensing enzyme FabF using additional malonyl-ACP substrates (Schujman et al., 2001). In summary, using the type II fatty acid synthase system B. subtilis can synthesise straight- or long-chain, saturated fatty acids of different lengths that serve as precursors for phospholipids.

1.4.2. Lipid composition and biosynthesis

The phospholipids in the membrane of exponentially growing B. subtilis 168 consist mainly of anionic phosphatidylglycerol (PG), around 40%, and zwitterionic phosphatidylethanolamine (PE), around 25% (Kawai et al., 2004). Other components found are positively charged lysylphosphatidylglycerol (LPG) and anionic cardiolipin (CL). These two latter compounds comprise 15.6 and 1.4% of the membrane, respectively. Phosphatidylinerine (PS), another phospholipid, is minimally found in exponentially growing cells. The rest ~20% of the plasma membrane is comprised of different forms of neutral glycolipids (GL) and other lipids (Kawai et al., 2004). During sporulation the relative levels of LPG drop in favour of CL and PG.

The biosynthesis of lipid in B. subtilis starts with the coupling of G3P to the generated acyl chains. G3P is on its turn synthesized from di-hydroxy-acetone-phosphate (DHAP) via the essential gene product of gpsA, glycerol-3-phosphate dehydrogenase (Fig. 1.8). Before the synthesized fatty acid can be linked to G3P the acyl-ACP needs to be activated to acyl-PO4 by acyltransferase PlsX (Paoletti et al., 2007). Then, membrane-bound acyltransferase PlsY, the product of the essential gene yneS, acylates the 1 position of G3P. Subsequently, PlsC (YhdO) converts the formed acyl-G3P into phosphatidic acid (PA), the central intermediate for all lipids (Lu et al., 2007, Paoletti et al., 2007, Zhang & Rock, 2008a). Neutral GL are formed by dephosphorylation of PA to diacylglycerol (DAG), which is then glycosylated with one or two glucose molecules from UDP-glucose (UDP-glc) by UDP-glucose diacylglycerol glucosyltransferase UgtP (Jorasch et al., 1998). The only essential phospholipid PG is produced in three steps from PA (de Mendoza et al., 2002). First, PA is activated to CDP-diacylglycerol (CDP-DAG) by essential synthase CdsA. Then, CDP-DAG is converted by PgsA into phosphatidylglycerol-phosphate (PG-Pi) through a condensation with G3P. Finally, anionic PG is formed by the removal of a phosphate group (Pi). Negatively charged CL (-2), is
Fig. 1.8. Phospholipid biosynthesis in *B. subtilis*. Essential enzymes are underlined. Gene names are shown in brackets when they differ from the enzyme name. End-products (phospho- and glycolipids) are shown in boxes. The central intermediate for all lipids phosphatidic acid (PA) is encircled. See the text for details. Abbreviations: DHAP: di-hydroxy-acetone-phosphate; G3P: sn-glycerol-3-phosphate; ACP: acyl carrier protein; PA: phosphatidic acid; DAG: diacylglycerol; GL: glycolipids; PS: phosphatidylyserine; PE: phosphatidylethanolamine; PG: phosphatidyglycerol; CL: cardiolipin; LPG: lysylphosphatidylglycerol.
formed by the condensation of two PG molecules. This reaction is mediated by cardiolipin synthase ClsA (YwnE), which is expressed during vegetative growth, or YwjE, involved in sporulation (Kawai et al., 2004). Cationic LPG on the other hand is synthesized from PG and a lysyl group, transferred form lysyl-tRNA by MprF (Multiple peptide resistant Factor) using lysyl-tRNA (Staubitz & Peschel, 2002). MprF is encoded by one gene, but its sequence was assigned yfiW and yfiX to due to a sequencing error (Kunst et al., 1997, Staubitz & Peschel, 2002). PS is obtained by PssA through the condensation with serine (de Mendoza et al., 2002). Finally, the most abundant lipid, PE, is synthesized via the decarboxylation of PS by Psd (Matsumoto et al., 1998).

The produced lipids in the discussed pathway all contain saturated acyl chains. Mono-unsaturated acyl chains can be generated from the previously synthesized lipids by oxygen-dependent desaturation. In B. subtilis this reaction is carried out by the gene product of des, the sole membrane-bound acyl-lipid desaturase (Aguilar et al., 1998). Des introduces a cis double bond at the 5-position of the acyl chains, thereby increasing the fluidity of the membrane (further discussed below).

1.4.3. Regulation of lipid biosynthesis and composition

The membrane of B. subtilis consists of complex lipids. As discussed above, they vary in length, degree of saturation, branching and net charge. Consequently, altering one or more components may lead to completely different properties (fluidity and net charge) of the membrane, affecting the permeability of hydrophobic molecules, active transport and protein interactions (Zhang & Rock, 2008b). Therefore, tight regulation is vital to maintain lipid homeostasis and to adapt to changing environmental (stress) conditions (Zhang & Rock, 2008a).

In B. subtilis FapR (fatty acid and phospholipid biosynthesis regulator) is the repressor of most genes of the type II fatty acid synthase system, itself (ylpC), and plsX and plsC (yhdO) of the first steps of phospholipid biosynthesis (Schujman et al., 2003). It was demonstrated that FapR is inhibited by one of the first intermediates of fatty acid biosynthesis malonyl-CoA (Schujman et al., 2003). Increasing concentrations of malonyl-CoA will cause derepression of the FapR-regulated genes. Therefore, malonyl-CoA serves as the regulator of the system. Cerulenin and triclosan, inhibitors of respectively FabF and FabI, did cause induction of the FapR regulon (Schujman et al., 2003). Additionally, a fabR (ylpC) mutant produced longer acyl chain than the wild-type strain, suggesting that FapR controls also the length and thereby the fluidity of the membrane. The regulation of ACC, the complex that catalyses the synthesis of malonyl-CoA (the first step of fatty acid biosynthesis generation), is not known. It was shown in E. coli that ACC expression is regulated by the growth rate and that AccB autoregulates accBC transcription (James & Cronan, 2004, Li & Cronan, 1993).
As discussed above, branched-chain amino acids are degraded by enzymes of the \textit{bkd} operon, regulated by BkdR, to generate branched-chain acyl-CoA primers. The operon is also negatively regulated by early-stationary-phase regulator CodY, global nitrogen regulator TnrA, and contains a promoter for SigL, involved in alternative carbon and nitrogen metabolism (Debarbouille et al., 1999). Furthermore, expression of \textit{sigL} is under carbon catabolite control of CcpA (Choi & Saier, 2005). Therefore, it is likely that the growth phase and nutrient availability regulate the fatty acid biosynthesis and the degree of branching.

Unsaturated acyl chains are generated by desaturase Des (Aguilar et al., 1998). The expression of \textit{des} is regulated by two-component system DesK/DesR and induced by a decrease in growth temperature (Mansilla & de Mendoza, 2005). Desaturation of the acyl-lipids will increase the membrane fluidity, which is needed for growth at lower temperatures. Since unsaturated fatty acids inhibit \textit{des} transcription at 37°C, it is thought that DesK is able to sense the membrane fluidity (Cybulski et al., 2002).

Besides the regulation of \textit{plsX} and \textit{plsC} (\textit{yhdO}) by FapR, the regulation of the phospholipid biosynthesis is not properly understood. Transcription of the \textit{pssA-ybfM-psd} operon is known to be mediated by SigA and SigX (Cao & Helmann, 2004). Induction of the SigX regulon will therefore increase the amount of neutral PE in the membrane and reduce the overall negative charge. In addition, extracytoplasmic function sigma factor SigX also regulates the \textit{dltABCDE-ywaA} operon, involved in the D-alanlylation of cell wall components lipiteichoic and wall teichoic acids (Perego et al., 1995). As a consequence, induction of the SigX regulon will result in a less negatively charged cell envelope. Since a \textit{sigX} mutant is more sensitive to cationic antimicrobial peptides, SigX is thought to be involved in the regulation of overall cell surface charge modification as a defence against cationic antimicrobial peptides (Cao & Helmann, 2004).

### 1.5. Outline of this thesis

The main goal of the work presented here is to reveal and understand the responses of \textit{B. subtilis} to weak organic acid stress and the resistance mechanisms involved. At the dawn of this research there was no data available on how this Gram-positive model-organism transcriptionally responds to this extensively used class of food preservatives. In addition, no clear information was at hand on the possible resistance mechanisms of this spore-forming organism. Thus, basically we tried to answer to following question: “\textit{What does }\textit{B. subtilis do to battle against stress caused by weak organic acids?”}

Chapter 1 presents the main player of this research: \textit{B. subtilis}. We shortly introduce its relevance to the food industry and discuss the relevant (generic) responses of this bacterium to different types of stress and starvation. Furthermore, the established knowledge on the modes of action of weak organic acids is explored and the available data on weak organic acid stress in other microorganisms are reviewed.
In Chapter 2 we set out to elucidate the time-resolved transcriptional responses of *B. subtilis* to the food preservative sorbic acid. The expression patterns are analyzed with the statistical tool T-profiler, recently developed in our lab (Boorsma *et al.*, 2005) and several mutants are tested on their susceptibility to sorbic acid stress.

An independent approach is used in Chapter 3 to identify genes important in sorbic acid stress adaptation. A mutant library is created using transposon mutagenesis and screened for strains encompassing an altered sensitivity or resistance to sorbic acid stress. The discovered mutants are further characterized by testing their susceptibilities to other related stresses.

A comparative physiological and transcriptional analysis of weak organic acid stress in *B. subtilis* is performed in Chapter 4. The minimal generic response to this type of stress is elucidated, as well as the specific and distinct responses involved.

In Chapter 5 a strain is constructed that is able to measure *in vivo* the intracellular pH of *B. subtilis*. The effect of weak organic acids and hyperosmotic shock is tested on the pH of the cytosol.

The aspects of heterogeneity in isogenic populations of *Bacillus* and its significance on the resistance of spores are discussed in Chapter 6. Shown are examples on how differences in sporulating conditions can affect the resistant properties of the spores and their capabilities to germinate and grow out.

Finally, Chapter 7 puts all newly acquired knowledge in perspective. The data is discussed, conclusions are made and suggestions are given on future experiments.

The data presented in this thesis can be used to search more specifically for targets in combating this common food spoiler. Hopefully this information can help with the discovery of new (natural) antimicrobial agents, as well as with more mild, however still safe, preservation techniques.