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MICROBIAL MECHANISMS OF TOLERANCE TO WEAK ACIDS: AN OVERVIEW

Topic Editors
Nuno Pereira Mira and
Miguel Cacho Teixeira

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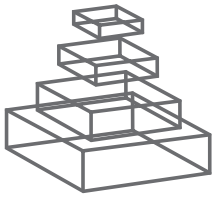

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MICROBIAL MECHANISMS OF TOLERANCE TO WEAK ACIDS: AN OVERVIEW

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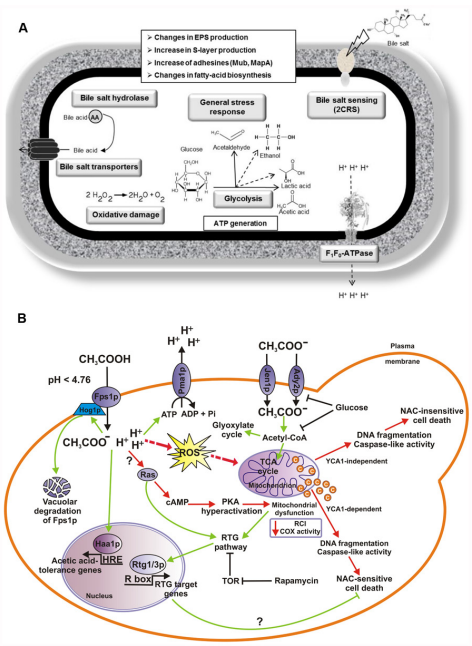

Schematic representation of the adaptive responses triggered by Lactobacilii and by Saccharomyces cerevisiae under stress induced by bile acids or by acetic acid, as reviewed in the articles of Ruiz et al., (2013) and Giannattasio et al. (2013) published in this e-book.

Ruiz L, Margolles A and Sánchez B (2013) Bile resistance mechanisms in Lactobacillus and Bifidobacterium. Front. Microbiol. 4:396. doi: 10.3389/fmicb.2013.00396

Giannattasio S, Guaragnella N, Ždralević M and Marra E (2013) Molecular mechanisms of Saccharomyces cerevisiae stress adaptation and programmed cell death in response to acetic acid. *Front. Microbio.* 4:33. doi: 10.3389/fmicb.2013.00033

Carboxylic acids are ubiquitous molecules found in microbial metabolic pathways and that have been explored for a wide array of applications including food preservation (e.g., acetic, propionic, benzoic, and sorbic acids), chemotherapy (e.g., the analgesic acetylsalicylic acid, the immunosuppressor mycophenolic acid or the antimalarial drugs artesunic and artemisinic acids) or agriculture (e.g., the herbicides 2,4-dichlorophenoxyacetic acid). This Research Topic contributes to the understanding of the molecular mechanisms underlying adaptation to weak acid stress in microbes, a knowledge base that impacts the fields of Medicine, Health, Food Safety and the Environment.

Table of Contents

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EDITORIAL
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Microbial mechanisms of tolerance to weak acid stress

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Thomas E. Hanson, University of Delaware, USA

Keywords: carboxylic acids, weak acid food preservatives, response and resistance to weak acid stress, food-borne spoilage microbes, tolerance to weak acids

Carboxylic acids are ubiquitous molecules found in microbial metabolic pathways and that have been explored for a wide array of applications including food preservation (e.g., acetic, propionic, benzoic, and sorbic acids), chemotherapy (e.g., the analgesic acetylsalicylic acid, the immunosuppressor mycophenolic acid or the antimalarial drugs artesunic and artemisinic acids) or agriculture (e.g., the herbicides 2,4-dichlorophenoxyacetic acid). This research topic contributes to the understanding of the molecular mechanisms underlying adaptation to weak acid stress in microbes [extensively reviewed in Piper et al. (2001); Boot et al. (2002); Cotter and Hill (2003); Smits and Brul (2005); Mollapour et al. (2008); Teixeira et al. (2011)], a knowledge base that impacts the fields of Medicine, Health, Food Safety, and the Environment.

The exploitation of carboxylic acids as "building-block molecules" for chemical synthesis has recently been a hot topic of research, accompanying the orientation of US and EU policy towards the development of biorefineries(Sauer et al., 2008; Abbott et al., 2009). To be economically sustainable biomass-based refineries must efficiently produce biofuels, but must also produce effective alternatives to the oil-derivatives that are used today as precursors/catalysts by the chemical industry. The carboxylic group, along with other functional groups frequently found in carboxylic acids, make these molecules attractive platforms for chemical synthesis and/or catalysis. The efforts put by the scientific community to develop efficient processes for large-scale production of carboxylic acids from renewable carbon sources, exploring different microbes as cell factories, have led to significant improvements (Sauer et al., 2008; Abbott et al., 2009); however, the yield of microbial carboxylic acid production is still limited. This low yield is mainly caused by to the toxic effects of the acids in the producing cells and by the diversion of substrate towards metabolites other than the acid of interest. The article of Jarboe et al. (2013) reviews the toxic effects exerted by, and the underlying adaptive responses to, lipophilic carboxylic acids in *Saccharomyces cerevisiae* and *Escherichia coli*, two host systems that have been exploited as cell factories. Within the same context, Steiger et al. (2013) review the most recent findings regarding biosynthesis of itaconic acid in *Aspergillus terreus* as well as the metabolic- and genetic engineering-based strategies attempted to improve the yield of production of this acid in this fungus and in other hosts.

The ability of microbial pathogens to colonize the human body is frequently dependent on their ability to tolerate carboxylic acids. This the case of probiotic bacteria that need to tolerate the presence of bile salts (composed by bile acids conjugated with glycine or taurine) to colonize the gut, or of *Candida* species that need to cope with acetic and lactic acids in the vaginal tract, produced by the bacteria that co-colonize that niche. Ruiz et al. (2013) review the main adaptive responses of probiotic Lactobacilli and Bifidobacteria to cope with the toxic effect exerted by bile salts and by bile. Several reports on adaptive mechanisms to weak acids in *Candida* are featured as well. The involvement of the multidrug (MDR) transporter of the Major Facilitator Superfamily (MFS) CgAqr1 in *C. glabrata* tolerance to acetic acid and to the antifungal drugs flucytosine and clotrimazole is herein demonstrated (Costa et al., 2013). The involvement of the *HOG1*-signalling pathway, controlled by the Hog1 protein kinase, in *C. glabrata* tolerance and response to sorbic acid is described by Jandric et al. (2013). Sorbic acid is widely used as a preservative in over-the-counter vaginal products and therefore the identification of genes/proteins involved in *C. glabrata* resistance to this compound is detrimental to improve the efficacy of those products and, consequently, to control infections caused by this pathogenic yeast.

Weak carboxylic acids (e.g., acetic, propionic, sorbic and benzoic acids) are important food preservatives and spoilage microbes must overcome their presence to grow in food products. Much of what is known today regarding the mechanisms underlying tolerance and resistance to weak acid food preservatives in spoilage Fungi, particularly at a genome-wide scale, was gathered in the experimental eukaryotic model yeast *Saccharomyces cerevisiae*, itself a spoilage yeast (Piper et al., 2001; Mollapour et al., 2008; Mira et al., 2010). Two studies dedicated to *S. cerevisae* responses to weak acid stress are included in this research topic. The review of Giannattasio et al. (2013) focuses on the equilibrium between the activation of pro-survival or pro-death mechanisms described to occur in acetic acid-stressed yeast cells. The second study, undertaken by Ullah et al. (2013), shows that under extreme weak acid stress *S. cerevisiae* cells prefer to preserve energy reserves thus limiting the activation of energy-consuming adaptive mechanisms (such as the activity of the plasma membrane proton pump). Results from Ullah et al. also reinforce a previous hypothesis that yeast cells adapt to weak acid stress by modifying the cell envelope to reduce the diffusion rate of the undissociated acid to the intracellular environment (Simoes et al., 2006; Mira et al., 2009). The study of Diakogiannis et al. (2013) shows that this mechanism of diffusional restriction is also relevant for protection against weak acid toxicity in the food-borne pathogen *Listeria monocytogenes*. The response of *Campylobacter jejuni*, also a food-borne pathogen, to formic acid is the scope

of the study performed by Kassem et al. (2013). Formic acid has been used as a feed additive to reduce emergence of *C. jejuni* in chickens, the primary reservoir of this pathogen. However, the data obtained suggest that formic acid might act as an inducer (or at least a positive modulator) of entry of *C. jejuni* cells into a viable-but-not-culturable (VBNC) state that allows these bacterial cells to become untraceable. The development of a pH-sensitive fluorescent probe to monitor the internal pH of *Bacillus subtillis* cells is the goal of the study of Van Beilen and Brul (2013). Using this newly developed probe the authors demonstrate that germination of *B. subtillis* spores involves a prominent increase in internal pH up to 7 (Van Beilen and Brul, 2013). In the presence of inhibitory concentrations was not altered of sorbic acid in the extracellular milieu reduced the germination rate and abrogated the increase in internal pH, while no significant effect was registered under acetic acid stress (Van Beilen and Brul, 2013). It thus seems that sorbic acid is more suited to prevent contamination of food products with *B. subtillis* than acetic acid.

Altogether this research topic highlights the importance of pursuing the in-depth study of the molecular mechanisms underlying the toxicity and resistance to weak organic acids as an important way to contribute for the development of more appropriate tools for the control and elimination of food-borne pathogens/contaminants that thrive in acidic environments and for the engineering of optimized strains to be used as superior cell factories, able to tolerate inhibitory weak acid concentrations, among other fermentation-related stresses.

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Understanding biocatalyst inhibition by carboxylic acids

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Carboxylic acids are an attractive biorenewable chemical in terms of their flexibility and usage as precursors for a variety of industrial chemicals. It has been demonstrated that such carboxylic acids can be fermentatively produced using engineered microbes, such as *Escherichia coli* and *Saccharomyces cerevisiae*. However, like many other attractive biorenewable fuels and chemicals, carboxylic acids become inhibitory to these microbes at concentrations below the desired yield and titer. In fact, their potency as microbial inhibitors is highlighted by the fact that many of these carboxylic acids are routinely used as food preservatives. This review highlights the current knowledge regarding the impact that saturated, straight-chain carboxylic acids, such as hexanoic, octanoic, decanoic, and lauric acids can have on *E. coli* and *S. cerevisiae*, with the goal of identifying metabolic engineering strategies to increase robustness. Key effects of these carboxylic acids include damage to the cell membrane and a decrease of the microbial internal pH. Certain changes in cell membrane properties, such as composition, fluidity, integrity, and hydrophobicity, and intracellular pH are often associated with increased tolerance. The availability of appropriate exporters, such as Pdr12, can also increase tolerance. The effect on metabolic processes, such as maintaining appropriate respiratory function, regulation of Lrp activity and inhibition of production of key metabolites such as methionine, are also considered. Understanding the mechanisms of biocatalyst inhibition by these desirable products can aid in the engineering of robust strains with improved industrial performance.

Keywords: tolerance, membrane damage, transporters, acid resistance, intracellular pH, biocatalyst robustness, carboxylic acid toxicity

INTRODUCTION

Carboxylic acids are useful biorenewable chemicals that can serve as precursors for drop-in replacements for petroleum-derived industrial chemicals (Mäki-Arvela et al., 2007; Lennen et al., 2010; Shanks, 2010; Carlos Serrano-Ruiz et al., 2012) and biologically-produced polymers (Wang et al., 2011) and alcohols (Perez et al., 2013). Much progress has been made in recent years in engineering workhorse biocatalysts, such as *Escherichia coli* and *S. cerevisiae*, for production of carboxylic acids (Lennen et al., 2010; Ranganathan et al., 2012; Zhang et al., 2012a,b), including recent reviews for both of these species (Abbott et al., 2009; Lennen and Pfleger, 2012; Liu and Jarboe, 2012).

However, in addition to our renewed interest in carboxylic acids as biorenewable chemicals, we have had a long history of using these compounds as food preservatives and soaps (Russell, 1991; Ricke, 2003; Kabara and Marshall, 2010). An even more ancient history involves the production of these compounds in the human digestive tract (Cummings and Macfarlane, 1991) and on human skin (Desbois and Smith, 2010). This functionality of carboxylic acids in inhibiting microbial activity presents a challenge in the microbial production of these compounds at a sufficiently high concentration and titer to enable an economically viable process.

Microbial inhibition by products or substrates is a relatively common problem in the production of biorenewable fuels and chemicals (Dunlop et al., 2011; Jarboe et al., 2011). This inhibitory action by carboxylic acids has already been cited as limiting performance in both *E. coli* (Lennen et al., 2011) and the cyanobacterium *Synechococcus elongatus* (Ruffing and Jones, 2012). The fact that *E. coli* strains have been developed that can produce 118 g/L (1.3 M) lactic acid and 83 g/L (0.70 M) succinic acid in defined minimal media demonstrates that organic acid tolerance can be increased by this organism (Jarboe et al., 2010). However, these two projects were enabled by the fact that production of the lactic and succinic acids were required to maintain redox and ATP balance, respectively, providing a selective marker for directed evolution (Jarboe et al., 2010). This is not possible in all cases and thus other strategies for increasing robustness are desired. It should be noted that titers of 0.78 M lactic acid have also been achieved in *S. cerevisiae* (Rossi et al., 2010). Information about the mechanism of inhibition can provide guidance to metabolic engineering strategies that increase microbial robustness (Dunlop et al., 2011; Jarboe et al., 2011; Wang et al., 2013) and thus enable a more economically viable and industrially relevant process (Chotani et al., 2000).

Here we provide a review of the current knowledge of the mechanisms of microbial inhibition by carboxylic acids. Since we are mainly interested in metabolic engineering for carboxylic acid production, we focus on *E. coli* and *S. cerevisiae*. Additionally, we mainly focus on straight-chain, saturated carboxylic acids of at least six carbons in length, such as hexanoic/caproic (C6:0), octanoic/caprylic (C8:0), decanoic/capric (C10:0), dodecanoic/lauric (C12:0), tetradecanoic/myristic (C14:0) and

hexadecanoic/palmitic (C16:0) acids. Given its abundance in biomass hydrolysate, acetic acid (C2:0) has been the focus of extensive research (Mills et al., 2009) and is described here only when there is demonstrated relevance to or lack of data for longer-chain acids. There have been several excellent previously reviews on carboxylic acids tolerance (Ricke, 2003; Desbois and Smith, 2010; Kabara and Marshall, 2010), and this work is intended to serve only as an overview of specific concepts and to provide insight for future studies, not a comprehensive review of all relevant studies.

CHARACTERIZATION OF INHIBITION AS A FUNCTION OF MOLECULE STRUCTURE AND MEDIA pH

The degree of inhibition by carboxylic acids can vary according to molecule identity (Kabara et al., 1972; Marounek et al., 2003; Kabara and Marshall, 2010), organism identity (Kabara et al., 1972), strain (DiezGonzalez and Russell, 1997) and growth condition (Viegas and Sa-Correia, 1995; Kasemets et al., 2006) and thus inhibitory concentrations are not listed here. A comparative study of inhibition by a weak acid (sorbic acid), an uncoupler (2,4-dinitrophenol) and a carboxylic acid (decanoic acid) observed that the carboxylic acid caused rapid cell death relative to the other two inhibitors and concluded that the mechanism of inhibition by carboxylic acids must be distinct from the other two molecule types (Stratford and Anslow, 1996). Desbois and Smith (2010) briefly review the association of molecule structure and shape with its potency as an inhibitor, but since most of these relationships deal with unsaturated molecules they are not discussed here. Our own studies have shown a significant increase in toxicity to *S. cerevisiae* on a molar basis as chain length increases from 6 to 8 to 10 carbons (Liu et al., 2013b), but this strong dependence on chain length was not observed with *E. coli* (Royce et al., 2013). The difference in the octanoic and decanoic responses in *S. cerevisiae* was also noted by a transcriptome-based study (Legras et al., 2010).

It is clear from the literature that carboxylic acid toxicity increases at lower pH values, particularly as the media pH nears the molecule pKa (Stratford and Anslow, 1996; Liu et al., 2013b; Royce et al., 2013). Another conserved factor of carboxylic acid toxicity is the link between toxicity and hydrophobicity (Zaldivar and Ingram, 1999), similar to the trends reported for solvent toxicity (Ramos et al., 2002). This relationship between toxicity, pKa and molecule hydrophobicity relates to transport of these molecules into the cell, as described in the following sections.

MOVEMENT IN AND OUT OF THE CELL

We are more interested in systems that produce carboxylic acids than those that are challenged by exogenously-supplied carboxylic acids. However, understanding the toxicity of these compounds first requires an understanding of how they enter the cell; the bulk of the currently-available data on this topic relates to exogenously supplied carboxylic acids. Carboxylic acids can pass through the cell membrane via diffusion or a transporter (Nikaido, 2003).

The distribution of carboxylic acids between their protonated (HA) and ionic forms (H^+ and A^-) is a function of the system pH and the molecule's pKa, as described by the Henderson–Hasselbalch equation

$$\mathrm{pH} = \mathrm{pKa} + \log \frac{[\mathrm{A}^-]}{[\mathrm{HA}]}$$

It has been shown that for model membranes the limiting step for membrane permeation is also a function of carboxylic acid chain length (Evtodienko et al., 1996). Specifically, for chain lengths of 2–6 carbons, transport through the membrane is limited by diffusion of the anion when the external pH is below the molecule pKa, but limited by diffusion of the neutral form when the pH is greater than the pKa (Evtodienko et al., 1996). For longer-chain carboxylic acids, transport through these model membranes is limited by the diffusion of the anionic form at all pH values (Evtodienko et al., 1996). Changes in membrane properties that increase tolerance to carboxylic acids are discussed below.

Generally speaking, diffusion of carboxylic acids follow Overton's Rule that membrane permeability is a function of molecule hydrophobicity (Al-Awqati, 1999; Kamp and Hamilton, 2006). Membrane permeability of the non-ionic form (P^m) was measured for a variety of monocarboxylic acids and related to the more readily-available hexadecane/water partition coefficient (K_p) with a correlation coefficient of 0.996 (Walter and Gutknecht, 1984) as

$$\log(P^m) = 0.90\ \log(K_p) + 0.89$$

Accumulation of the anions within the cell has been asserted as one of the main mechanisms of microbial inhibition by carboxylic acids (Carpenter and Broadbent, 2009). The magnitude of this accumulation is a function of the external anion concentration and external pH (Carpenter and Broadbent, 2009); the biological implication of this accumulation is discussed below. Carpenter and Broadbent's (2009) conclusions are consistent with Evtodienko's et al. (1996) results for carbon chains of six carbons of less. The reason for the differences regarding longer-chain molecules is not clear.

There has been more success in identification of carboxylic acid transporters in *S. cerevisiae* than in *E. coli*. The Pdr12 ABC transporter was originally discovered during a study of sorbic (2,4-hexadienoic) acid toxicity and was shown to contribute to organic acid tolerance through the energy-dependent removal of carboxylate anions from the cell interior (Piper et al., 1998). Presumably Pdr12 is the transporter proposed to be necessary for acquisition of octanoic acid tolerance in other studies (Cabral et al., 2001), as it has since been shown to contribute to octanoic acid tolerance in *S. cerevisiae*, along with the Tpo1p transporter (Legras et al., 2010). The transporter-encoding *AQR1* gene has been shown to provide protection of *S. cerevisiae* against carboxylic acids of six carbons or less, but does not provide protection against octanoic acid (Tenreiro et al., 2002). Given the recent successes in increasing biocatalyst performance by provision of the appropriate product exporter (Dunlop et al., 2011; Park et al., 2011), this is an area that could possibly benefit from increased attention.

MEMBRANE DAMAGE

Permeability of the cell membrane to carboxylic acids is indicative of the solubility of these compounds in this vital structure. The

damage caused to the cell membrane has been presented as one of the main mechanisms of microbial inhibition by carboxylic acids (Ricke, 2003; Desbois and Smith, 2010). A recent nanoscale imaging study of membrane disruption by antimicrobial peptides was able to visualize formation of membrane pores and their expansion to the point of membrane disintegration (Rakowska et al., 2013), though it is not yet clear whether carboxylic acid membrane damage proceeds in this manner.

A recent omics-wide study of an *E. coli* strain engineered to produce a mixture of C8–C14 carboxylic acids to a total titer of approximately 300 mg/L concluded that membrane stresses are one of the major challenges faced by this strain (Lennen et al., 2011). Membrane stress was evidenced by increased permeability of the inner membrane to a nucleic acid dye and an 85% decrease in cell viability associated with carboxylic acid production, where cell viability was quantified by colony forming units relative to the non-producing strain in the same condition (Lennen et al., 2011). It was also noted that membrane damage associated with carboxylic acid production was increased relative to challenge with exogenously-supplied carboxylic acids (Lennen et al., 2011).

A transcriptional study of the conserved weak organic acid response in *S. cerevisiae* during anaerobic growth concluded that many of the genes activated in response to benzoate, sorbate, acetate, and propionate are related to cell wall structure and organization (Abbott et al., 2007). Our own transcriptome analysis of exogenous challenge of *S. cerevisae* with 43 mg/L (0.30 mM) octanoic acid at pH 5.0 and 30°C also concluded that membrane damage was the most significant effect (Liu et al., 2013b). Further studies were performed using Mg^{2+} as a representative small molecule that should be retained within the cell, but leaks out of damaged cell membranes. Mg^{2+} leakage was observed to increase in a dose-dependent manner in response to exogenously supplied C8 and in an increasing response to chain length when challenged with 0.30 mM hexanoic, octanoic or decanoic acids (Liu et al., 2013b). We detected no change in membrane fluidity or hydrophobicity (Liu et al., 2013b). A short period of adaptation to octanoic acid resulted in increased resistance to membrane damage, as evidenced by decreased Mg^{2+} efflux. Note that the importance of maintaining appropriate membrane fluidity and methods for its characterization have been reviewed elsewhere (Marguet et al., 2006). The mechanisms of this adaptation and the accompanying changes in membrane lipid composition are discussed below.

In *E. coli*, challenge with octanoic acid in minimal media at pH 7.0 and 37°C resulted in both a significant decrease of membrane polarization, indicative of an increase in fluidity, and Mg^{2+} leakage at levels approximately 50% of those observed with chloroform treatment (Royce et al., 2013). However, after a short period of adaptation to octanoic acid, cells became resistant to its fluidizing effect but not the membrane damage evidenced by Mg^{2+} efflux. During this adaptation, the membrane lipid composition changed, as discussed below, and the cell surface hydrophobicity significantly decreased (Royce et al., 2013). Consistent with this data, our analysis of a carboxylic acid-producing strain showed that membrane leakage, but not fluidity, increased as the carboxylic acid titer increased (Royce et al., 2013). This strain produced predominantly tetradecanoic and palmitic acids (Ranganathan et al., 2012) to a final titer of 600 mg/L and was characterized in minimal media at 30°C.

It should be noted that when Zaldivar and Ingram (1999) tested the sensitivity of *E. coli* to various organic acids, including hexanoic acid, well above concentrations that inhibits growth by 80%, only moderate amounts of Mg^{2+} leakage were detected and the authors concluded that membrane damage was not a key component of organic acid toxicity (Zaldivar and Ingram, 1999). This difference could possibly be due to the fact that these authors were studying an ethanol-producing *E. coli* strain in rich media.

This damage to the cell membrane can not only impact retention of valuable metabolites, such as Mg^{2+}, but can also impact membrane-associated cell functions. Systems with damaged membranes frequently show evidence of oxidative stress, possibly due to decreased function of the electron transport chain (Lennen et al., 2011; Segura et al., 2012). Recent studies and reviews of carboxylic acid toxicity in *S. cerevisiae* have noted the link between toxicity and production of reactive oxygen species (ROS; Abbott et al., 2009; Legras et al., 2010). A thorough black box metabolic characterization was performed regarding octanoic acid toxicity with a strain of *E. coli* engineered to produce octanoic acid from octane (Rothen et al., 1998). It was shown that pulses of octanoic acid during growth in defined media resulted in transient decreases in production of CO_2 and biomass, decreased utilization of glucose and O_2 and increased production of acetate (Rothen et al., 1998), leading this author to propose that this is evidence of decreased aerobic respiration, possibly due to damage of the membrane-associated electron transport chain.

CHANGES IN MEMBRANE PROPERTIES TO INCREASE TOLERANCE

As described above, a short period of adaptation to carboxylic acids can enable changes that increase tolerance to these inhibitory compounds. Understanding these changes can enable metabolic engineering strategies for increased tolerance. The extensive knowledge regarding membrane-related solvent toxicity may also be of use here (Ramos et al., 2002; Segura et al., 2012).

For example, it has been shown that mutant strains with decreased cell surface hydrophobicity have increased organic solvent tolerance (Aono and Kobayashi, 1997). This decrease in hydrophobicity was attributed to an increase in lipopolysaccharide content; lipopolysaccharide amino acid composition was unchanged (Aono and Kobayashi, 1997). Our own studies have shown that *E. coli* cell surface hydrophobicity decreases during adaptation to octanoic acid (Royce et al., 2013). Solvent tolerance is also frequently attributed to changes in the saturated/unsaturated ratio, *cis*/*trans* isomerization, the length of acyl chains, phospholipid head groups, lipopolysaccharide composition and membrane hydrophobicity (Ramos et al., 2002; Segura et al., 2012). Understanding the genetic and molecular mechanisms of these changes and their role in increasing tolerance can guide engineering efforts.

The most frequently-noted changes in response to carboxylic acid challenge, either exogenously supplied or during production, deal with the composition of the membrane lipids. For example, Lennen et al., 2011 study of an *E. coli* strain that produces free fatty acids noted an increase in the long-chain unsaturated fatty

acid content in the cell membrane. Our own studies of *E. coli* MG1655 following 3 h of adaptation to octanoic acid at pH 7.0 showed a significant increase in average lipid length and a significant decrease in the saturated/unsaturated ratio (Royce et al., 2013). Our studies of octanoic adaptation of *S. cerevisiae* at pH 5.0 showed a similar, significant increase in average lipid length (Liu et al., 2013b). The saturated/unsaturated ratio was not as clear, showing a significant increase at moderate inhibitory octanoic acid concentrations of 43 and 72 mg/L (0.30 and 0.50 mM), but no significant difference between the control samples and those adapted to 115 mg/L octanoic acid (0.8 mM), a concentration which decreases the specific growth rate by more than 90% (Liu et al., 2013b).

The question remains as to whether this change in membrane composition is a microbial strategy for increasing carboxylic acid tolerance or a side effect of the presence of carboxylic acid and thus its own mechanism of inhibition. Lennen and Pfleger (2013) hypothesized that the decreased saturated fatty acid content was a mechanism of carboxylic acid toxicity and engineered their carboxylic acid-producing *E. coli* strain in order to restore the saturated fatty acid content to normal levels. Their engineering strategy was successful in increasing the saturated fatty acid content during carboxylic acid production, though levels were still higher than that observed for the non-producing control strain. In support of their hypothesis, viability of the carboxylic-acid producing strains was significantly increased in the strain engineered for increased saturated fatty acid content (Lennen and Pfleger, 2013). Similar results were observed when saturated fatty acid content was increased in order to increase *E. coli* ethanol tolerance (Luo et al., 2009). These results suggest that the presence of carboxylic acids precludes *E. coli* from maintaining the appropriate amount of saturated fatty acids in the cell membrane, leading to decreased viability.

Contrastingly, our research team interpreted the association between increased oleic acid (C18:1) content in the *S. cerevisiae* cell membrane after short-term adaptation to octanoic acid and increased resistance to membrane damage and growth inhibition by octanoic acid as evidence that increasing the oleic acid content in the membrane is beneficial for carboxylic acid tolerance (Liu et al., 2013b). We found that supplementing the growth media with 1.0 mM oleic acid increased the C18:1 content in the membrane to 54% (by area), relative to the 22% observed in the control cells and 35% in the cells adapted to 0.5 mM octanoic acid. This increased oleic acid content was accompanied by a significant decrease in octanoic acid-mediated Mg^{2+} leakage and decreased growth inhibition by 0.5 and 1.0 mM octanoic acid (Liu et al., 2013b). Subsequent metabolic engineering efforts were successful in increasing the oleic acid content independent of media supplementation, but not to the level needed for increased robustness (Liu et al., 2013b). Thus, at this point it is not clear whether there can be a general conclusion about either increasing or decreasing saturated fatty acid content as a means of increasing carboxylic acid robustness.

In addition to consistent reports of increased unsaturated fatty acid content in *E. coli* during carboxylic acid challenge or production, there have been consistent reports of increased cyclopropane fatty acid content (Lennen and Pfleger, 2013; Royce et al., 2013). The most common cyclopropane fatty acid in *E. coli* is C17:1, also refered to as C17cyc, produced by methylation of C16:1 phospholipids by the Cfa enzyme. Cyclopropane fatty acids have been demonstrated as very important to membrane permeability to protons and thus to survival in acidic conditions (Chang and Cronan, 1999; Shabala and Ross, 2008). However, engineering of *S. cerevisiae* to contain up to 10% (by area) C17cyc in the membrane was also not helpful for carboxylic acid tolerance (Liu et al., 2013b). The results support the proposition that it is transport of the anion and neutral forms of the carboxylic acid, and not the proton, that is problematic for microbial growth.

The cell membrane contains more than just phospholipids. Alterations in the abundance or structure of other membrane components can also impact carboxylic acid sensitivity. Disruption of ergosterol content in *S. cerevisiae* membranes via deletion of *erg4* increased sensitivity to undecanoic (C11:0), 10-undecanoic (C11:1Δ10) and dodecanoic acids (McDonough et al., 2002). Note that ergosterol is 22-carbon sterol that typically accounts for more than 60% of the *S. cerevisiae* sterol content (McDonough et al., 2002). We were unable to identify any reports of attempts to increase carboxylic acid tolerance via increases of ergosterol content, though it has been shown that exogenous ergosterol supplementation increases tolerance to the cyclic terpene hydrocarbon limonene (Liu et al., 2013a).

The lipopolysaccharide leaflet on the outer membrane provides a substantial barrier to diffusion; the diffusion of hydrophobic steroid probes was shown to be two orders of magnitude slower through this leaflet than through model phospholipid bilayer membranes (Nikaido, 2003). However, this means that mutations or defects that disrupt this leaflet, resulting in the "deep rough" phenotype enable increased vulnerability to compounds that enter the cell primarily through diffusion (Nikaido, 2003). Changes in the lipopolysaccharide structure, such as deletion of certain side chains, can also increase weak acid sensitivity (Barua et al., 2002; Nikaido, 2003). This is a tantalizing area of focus for engineering tolerance to carboxylic acids.

INTRACELLULAR ANION ACCUMULATION

Transport of carboxylic acids into the cell interior, and presumably their accumulation during production, can have a variety of effects on cellular processes. Acidification of the cell interior has been recognized as a key effect of carboxylic acids (Ricke, 2003). This acidification can occur, for example, when the non-ionic HA form enters the cell and then dissociates into H^+ and A^- ions.

Treatment with 56 mg/L (0.39 mM) octanoic acid resulted in a drop of intracellular pH to below 5.5 for approximately 80% of *S. cerevisiae* cells at 30°C, pH 4.0 (Viegas et al., 1998). Contrastingly, only 30% of cells had an intracellular pH below 5.5 in the control condition. In addition to the potential inhibition of enzymatic processes at this low pH, this acidification imposes a metabolic burden through the use of the ATP-dependent ATPase enzyme to remove the excess protons (Viegas et al., 1998). Studies of the relationship between temperature, carboxylic acid toxicity and intracellular pH noted that toxicity varied with temperature while intracellular pH did not, and thus the authors concluded that toxicity is not totally explained by the decrease in intracellular pH (Viegas and Sa-Correia, 1995). Transcriptome analysis

of carboxylic acid-producing *E. coli* strains showed evidence of acid stress when analyzed in shake flask cultures, but not during growth in controlled fermentors (Lennen et al., 2011). This difference could possibly be due to differences in oxygen availability, as oxidative phosphorylation may be needed in order to produce the ATP needed for proton export, and other stress response components.

In addition to burdens imposed by excess protons, carboxylic anions can accumulate to high concentrations, an effect that has mainly been studied in regards to acetic acid. For example, *E. coli* K12 strains accumulated up to 30 g/L (500 mM) internal acetate during challenge with 4.8 g/L (80 mM) exogenous acetic acid (DiezGonzalez and Russell, 1997). Other possibly problems associated with anion accumulation include changes in cell turgor (Comte et al., 2007) and disruption of key amino acid pools (Roe et al., 2002).

Just as there are possible changes in cell membrane properties that can increase resistance to carboxylic acids, there are changes that can help mitigate anion accumulation. Specifically, it has been shown that when challenged with 4.8 g/L (80 mM) acetate at pH 5.9, the K12 *E. coli* strain maintains an intracellular pH of 6.8 and accumulates up to 30 g/L (500 mM) intracellular acetate. Contrastingly, the acid-tolerant *E. coli* O157:H7 maintains an intracellular pH of 6.1 and accumulates only 18 g/L (300 mM) intracellular acetate even when external acetate concentrations are as high as 9.6 g/L (160 mM) (DiezGonzalez and Russell, 1997). The authors of this study concluded that this ability to withstand and maintain a lower intracellular pH, as well as the production of excess D-lactate, decreased the driving force for carboxylic acid transport and thus the magnitude of anion accumulation (DiezGonzalez and Russell, 1997), consistent with the conclusion of an earlier study (Russell, 1992). The genetic elements driving these differences remain unclear, but are attractive targets for future engineering efforts. Insight provided by extensive studies of *E. coli* survival in acidic conditions may provide insight (Foster, 2004).

OTHER EFFECTS

Many transcriptome-based studies have noted activation of oxidative stress response genes during production of or challenge with various carboxylic acids (King et al., 2010; Legras et al., 2010; Lennen et al., 2011; Ruenwai et al., 2011). Further tests in *S. cerevisiae* have confirmed not only increased production of reactive oxygen species in these conditions (Teixeira et al., 2004; Cipak et al., 2008; Ruenwai et al., 2011), but also increased activity of ROS-scavenging catalase and superoxide dismutase enzymes (Teixeira et al., 2004; Cipak et al., 2008). This increased abundance of ROS in yeast has largely been attributed to damage of the cell membrane and/or damage to the mitochondrial membrane (Mollapour et al., 2008; King et al., 2010; Lennen et al., 2011; Ruenwai et al., 2011). Damage to the mitochondrial membrane not only results in decreased function of the mitochondrial respiratory chain, but can also result in mutagenesis of the vulnerable mitochondrial DNA (Piper, 1999).

There are presumably other metabolic problems imposed by carboxylic acids, both in their neutral and ionic forms, that accumulate during exogenous challenge or production. The bulk of studies at this time have focused on the most apparent targets: the

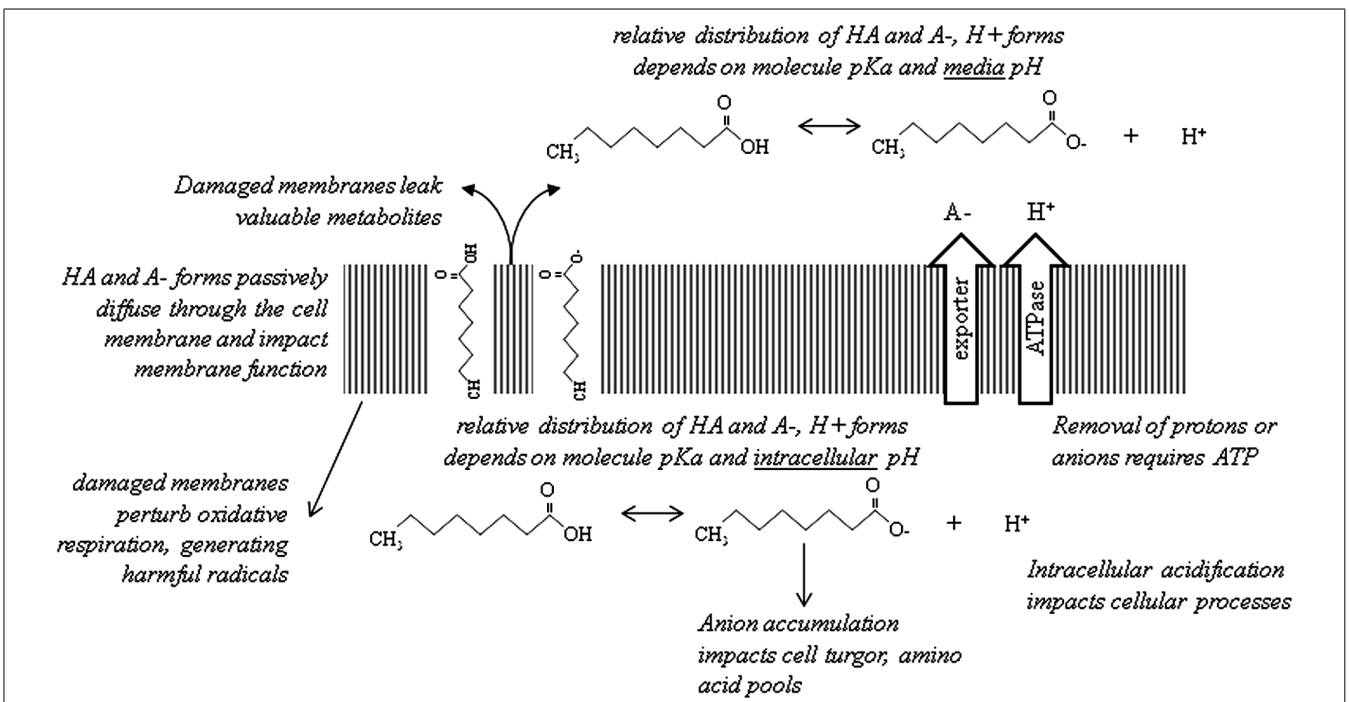

FIGURE 1 | Summary of the major mechanisms of carboxylic acid toxicity discussed in this article. For simplicity, the cell wall/membrane(s) are shown as a single structure. Octanoic acid (C8) is used as a representative carboxylic acid.

cell membrane, anion accumulation and cytoplasm acidification. However, there are hints of other effects. For example, it has been suggested that butyrate (C4:0) interacts directly with the leucine-responsive Lrp molecule (Nakanishi et al., 2009; Tobe et al., 2011), presumably due to structural similarities between butyrate and leucine. Thus far, these studies have mainly been motivated by the role of butyrate in regulating virulence. However, Lrp is a global regulator of *E. coli* metabolism (Yokoyama et al., 2006) and this interaction, if it extends to longer-chain carboxylic acids, could have a significant impact on the metabolism of producer strains.

Accumulation of carboxylate anions could increase the ionic strength of the cell interior, potentially inhibiting the activity of enzymes such as homocysteine transmethylase (MetE; Whitfield et al., 1970), an enzyme required for methionine biosynthesis. Intriguingly, accumulation of the MetE precursor homocysteine, which is itself toxic, has been shown to occur during acetate-mediated growth inhibition (Roe et al., 2002). This inhibition of MetE, and other ionic strength- or pH-sensitive enzymes, may have long-reaching effects on the biocatalyst metabolism.

Problems such as these may not yet be apparent due to "masking" by responses to membrane damage and acid stress. Once these primary problems are addressed, other metabolic problems may be detected.

CONCLUSION

Carboxylic acids are a tantalizing class of biorenewable chemicals, but it appears that their toxicity is currently limiting further advances in biocatalyst performance. Toxicity is largely related to membrane damage, but additional metabolic effects warrant further investigation. However, the assertion that addressing toxicity could improve biocatalyst performance is tempered by Lennen and Pfeger's (2013) finding that metabolic engineering strategies that increased carboxylic acid tolerance did not result in increased carboxylic acid titers. It is not yet clear if other strategies to improve robustness to these compounds will actually enable improved biocatalyst performance in terms of yields, titers and productivities.

Here we have briefly reviewed the current knowledge regarding carboxylic acid toxicity (**Figure 1**) and attempts to increase tolerance. A recent review of *E. coli*-based carboxylic acid production proposed engineering of carboxylic acid exporters and regulation of membrane composition as two key areas for future study (Lennen and Pfleger, 2012). We agree wholeheartedly with these suggestions. Most pressing is the need to address membrane damage; such work would be beneficial for solvent tolerance as well. It is not yet clear if intracellular acidification is a problem for carboxylic acid-producing strains. Other metabolic burdens associated with production of these compounds should become apparent as currently-known problems are addressed.

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Biochemistry of microbial itaconic acid production

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Itaconic acid is an unsaturated dicarbonic acid which has a high potential as a biochemical building block, because it can be used as a monomer for the production of a plethora of products including resins, plastics, paints, and synthetic fibers. Some *Aspergillus* species, like *A. itaconicus* and *A. terreus*, show the ability to synthesize this organic acid and *A. terreus* can secrete significant amounts to the media (>80 g/L). However, compared with the citric acid production process (titers >200 g/L) the achieved titers are still low and the overall process is expensive because purified substrates are required for optimal productivity. Itaconate is formed by the enzymatic activity of a *cis*-aconitate decarboxylase (CadA) encoded by the *cadA* gene in *A. terreus*. Cloning of the *cadA* gene into the citric acid producing fungus *A. niger* showed that it is possible to produce itaconic acid also in a different host organism. This review will describe the current status and recent advances in the understanding of the molecular processes leading to the biotechnological production of itaconic acid.

Keywords: *cis*-aconitic acid decarboxylase, *Aspergillus terreus*, *Aspergillus niger*, metabolic engineering, biochemical pathways, microbial organic acid production, industrial microbiology

INTRODUCTION

Itaconic acid (2-methylidenebutanedioic acid) is an unsaturated di-carbonic acid. It has a broad application spectrum in the industrial production of resins and is used as a building block for acrylic plastics, acrylate latexes, super-absorbents, and anti-scaling agents (Willke and Vorlop, 2001; Okabe et al., 2009). Since the 1960s the production of itaconic acid is achieved by the fermentation with *Aspergillus terreus* on sugar containing media (Willke and Vorlop, 2001). Although also other microorganisms like *Ustilago zeae* (Haskins et al., 1955), *U. maydis*, *Candida* sp. (Tabuchi et al., 1981), and *Rhodotorula* sp. (Kawamura et al., 1981) were found to produce itaconic acid, *A. terreus* is still the dominant production host, because so far only bred strains of this species can reach levels of up to 80–86 g/L (Okabe et al., 2009; Kuenz et al., 2012). Since the 1990s, itaconic acid as a renewable material is attracting a lot of interest. Although the production costs for itaconic acid are declining in the last years ($ 4 per kg in 2001; Willke and Vorlop, 2001), it is still a valuable product with an estimated price of $ 2 per kg. Currently, the worldwide production capacity of itaconic acid is expected to be about 50 kt per year, facing a demand of about 30 kt (Shaw, 2013, Itaconix Corporation, personal communication). Especially, for the production of polymers it is of interest, because in the future it can function as a substitute for acrylic and methacrylic acid used for the production of plastics (Okabe et al., 2009). However, these applications require an even lower price of the starting material. The current knowledge about the biotechnological production of itaconic acid was recently reviewed (Willke and Vorlop, 2001; Okabe et al., 2009). The latter review covers the industrial production of itaconic acid and the applications of this product. Therefore, we focus in this report on the recent advances with an emphasis on the biochemistry of the process and new genetic engineering targets. For rational strain improvement, it is essential to understand the underlying biological concepts and biochemical pathways leading to the production of this important organic acid in microorganisms.

BIOSYNTHESIS PATHWAY

Kinoshita (1932) recognized that a filamentous fungus was able to produce itaconic acid and consequently described this species as *A. itaconicus*. The biosynthesis of itaconic acid was for a long time hotly debated, because it was not clear whether itaconic acid arises from a pathway including parts of the tricarboxylic acid (TCA) cycle or an alternative pathway via citramalate or the condensation of acetyl-CoA.

Bentley and Thiessen (1957a) proposed a pathway for the biosynthesis of itaconic acid, which is depicted in **Figure 1**. Starting from a sugar substrate like glucose the carbon molecules are processed via glycolysis to pyruvate. Then the pathway is split and part of the carbon is metabolized to Acetyl-CoA releasing a carbon dioxide molecule. The other part is converted to oxaloacetate so that the previously released carbon dioxide molecule is again incorporated. In the first steps of the citric acid cycle, citrate and *cis*-aconitate are formed. In the last step, the only itaconic acid pathway dedicated step, *cis*-aconitate decarboxylase (CadA) forms itaconic acid releasing carbon dioxide. This pathway was confirmed by tracer experiments with ^{14}C and ^{13}C labeled substrates (Bentley and Thiessen, 1957a; Winskill, 1983; Bonnarme et al., 1995) and also the necessary enzymatic activities have been all determined (Jaklitsch et al., 1991).

The formation of carboxylic acids, like citric and itaconic acid, involves the shuttling of intermediate metabolites between different intracellular compartments and utilizes the different enzymatic capabilities of the respective compartment. In case

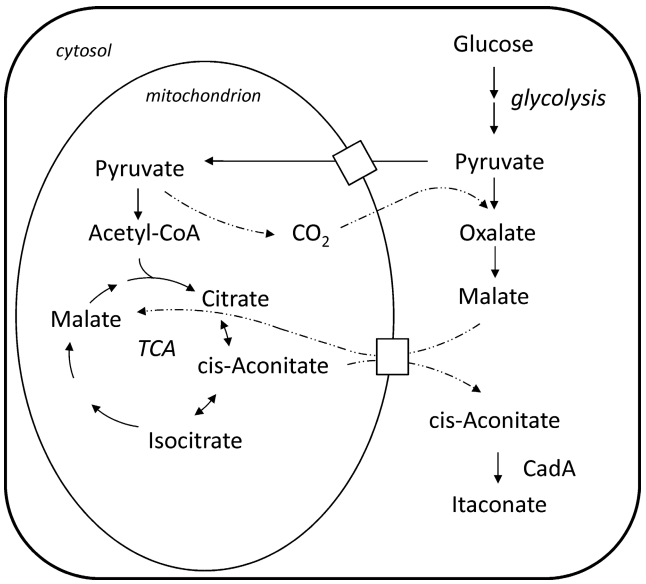

FIGURE 1 | Biosynthesis pathway of itaconate and its compartmentalization between cytosol and mitochondrion in the *A. terreus* cell; note: *cis*-aconitate transport is speculative.

of itaconic acid the compartmentalization of the pathway was analyzed by fractionized cell extracts distinguishing the enzymatic activity of a mitochondrial from a cytosolic enzyme. It was found that the key enzyme of the pathway, CadA, is not located in the mitochondria but in the cytosol (Jaklitsch et al., 1991), whereas the enzymes preceding in the pathway, namely citrate synthase and aconitase, are found in the mitochondria. However, a residual level of aconitase and citrate synthase activity is also found in the cytosolic fraction. The proposed mechanism is that *cis*-aconitate is transported via the malate–citrate antiporter into the cytosol (Jaklitsch et al., 1991). However, so far it was not shown whether *cis*-aconitate makes use of the mitochondrial malate–citrate antiporter or uses another mitochondrial carrier protein to be translocated to the cytosol.

Besides *A. terreus*, itaconic acid is known to be produced also by other fungi like *U. zeae* (Haskins et al., 1955), *U. maydis* (Haskins et al., 1955; Klement et al., 2012), *Candida* sp. (Tabuchi et al., 1981), and *Rhodotorula* sp. (Kawamura et al., 1981). No further investigations exist about the underlying reaction principles leading to itaconic acid formation in those species. However, recent evidence (Strelko et al., 2011; Voll et al., 2012) points into the direction that CadA activity constitutes the general pathway toward the formation of itaconic acid in nature. Very recently, itaconic acid was detected in mammalian cells, where it was found in macrophage-derived cells (Strelko et al., 2011). Those cells also possess a CadA activity and have the ability to form itaconic acid *de novo*. But, up to now no specific gene encoding this enzymatic activity was identified in mammalian cells.

However, the physiological role of itaconic acid in mammalian cells is still unknown. Strelko et al. (2011) speculate on the role of itaconic acid as an inhibitor of metabolic pathways, because it is described as an enzymatic inhibitor. On the one hand, itaconic acid is known to inhibit isocitrate lyase (Williams et al., 1971; McFadden and Purohit, 1977), which is the crucial part of the glyoxylate shunt, and thus can act as an antibacterial agent. On the other hand, itaconic acid can inhibit fructose-6-phosphate 2-kinase (Sakai et al., 2004) and thus have a direct influence on the central carbon metabolism. In rats it was shown that a itaconate diet leads to a reduced visceral fat accumulation, because of a suppressed glycolytic flux (Sakai et al., 2004).

ITACONIC ACID PATHWAY SPECIFIC ENZYMES AND GENES

The reaction catalyzed by the *cis*-aconitic acid decarboxylase was already described in 1957 (Bentley and Thiessen, 1957a,b). Subsequently performed ^{13}C and ^{14}C labeling experiments (Winskill, 1983; Bonnarme et al., 1995) confirmed the reaction scheme depicted in **Figure 2**. Itaconic acid is formed by an allylic rearrangement and decarboxylation from *cis*-aconitic acid removing either carbon C1 or C5 from the starting citric acid molecule (because of the symmetry of the molecule).

Furthermore, certain properties of the *A. terreus* CadA enzyme were determined: it has a K_m value of 2.45 mM (37°C, pH 6.2) and a pH optimum of 6.2 (Dwiarti et al., 2002). At pH 7.5 the activity drops significantly and is below 20% of the maximal value (Dwiarti et al., 2002). Until 2008, the sequence of the CadA protein was unknown, because the protein exhibits a general low stability. Kanamasa et al. (2008) were able to purify a substantial amount of the enzyme. By sequencing of the protein the N-terminal and four internal sequences were determined, which produced a single hit, ATEG_09971, in the genome database of *A. terreus*. The gene was named *cad1* and its protein product CadA. However, according to the nomenclature guidelines for *Aspergillus* it should rather be named *cadA* and CadA. The activity of the enzyme as a *cis*-aconitic acid decarboxylase (EC 4.1.1.6) was confirmed after heterologous expression of the gene in *Saccharomyces cerevisiae*. The CadA protein is a 490 amino acid protein (55 kDa) and has a high sequence identity with proteins from the MmgE/PrpD family, which includes 2-methylcitrate dehydratases. However, it is not clear, whether CadA has also a 2-methylcitrate dehydratase activity or whether a family member of the MmgE/PrpD class has also an activity as a *cis*-aconitic acid decarboxylase.

In contrast to the enzyme purification strategy, Li et al. (2011) used a transcriptomic approach to identify the *cadA* gene. A clone of the *A. terreus* strain NRRL1960 was cultivated at different conditions (pH, dissolved oxygen, etc.), which yielded different productivities and titers for itaconic acid. The conditions, which exhibited the highest difference in productivity and titer, were transcriptionally analyzed on a microarray with the assumption that genes involved in the itaconic acid pathway show an altered (higher) expression level during producing conditions. The *cadA* gene was highly scored in this analysis and thus can be identified in such an analysis. Interestingly another gene, encoding a mitochondrial carrier protein, was also highly scored in this analysis. This gene is located directly upstream of the *cadA* gene on the genome in *A. terreus*. Downstream of the *cadA* gene another transporter can be found which is annotated as a putative Major Facilitator Superfamily transporter. The mitochondrial carrier protein was detected in the transcriptomic analysis and was shown to have a direct positive influence on the itaconic acid production (Jore et al., 2011;

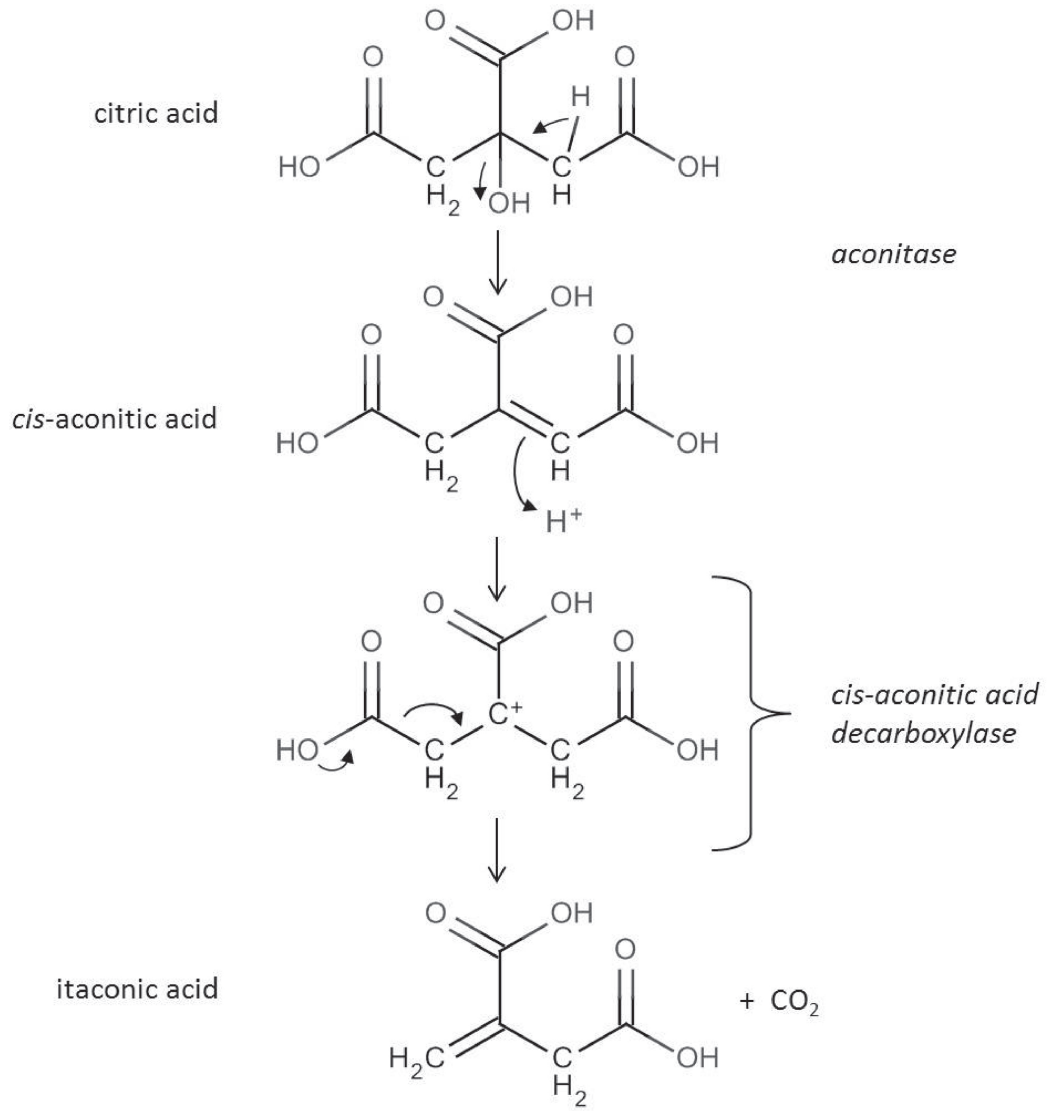

FIGURE 2 | In the citric acid cycle *cis*-aconitic acid is formed as an intermediate during the conversion of citric acid to isocitric acid. *cis*-aconitic acid is decarboxylated by the CadA enzyme to itaconic acid releasing CO_2 (Bentley and Thiessen, 1957b).

van der Straat et al., 2012). However, the mechanism and substrates of this putative transporter are still unknown and its role needs to be clarified, but it can be speculated that intermediates of the biosynthesis pathway like *cis*-aconitic acid are transported with this protein.

The activity of the *cis*-aconitic acid decarboxylase is crucial for the performance of the whole itaconic acid biosynthesis pathway. In an itaconic acid overproducing strain, which was obtained by an selection on high itaconate levels (Yahiro et al., 1995), five times higher transcription levels of the *cadA* gene were found than in a comparable wild type strain but no change in the amino acid sequence was detected (Kanamasa et al., 2008). Expressing the *cadA* gene in *A. niger* under various constitutive promoters of different expression strength demonstrated that the itaconic acid productivity directly correlates with the *cadA* transcript level (Blumhoff et al., 2013). It can be concluded that a high transcriptional level of this gene is essential for an optimal production performance. A high transcriptional level of the gene might be necessary, because of a low stability of the enzyme *in vivo*, which was found to be rather unstable *in vitro* (Dwiarti et al., 2002; Kanamasa et al., 2008).

CATABOLIZATION OF ITACONIC ACID

Much is known about the biosynthesis of itaconic acid and the underlying enzymatic mechanisms, but for a complete biochemical picture of a certain metabolite, also the knowledge about its degradation is necessary. Unfortunately, the information about the degradation pathway of itaconic acid is scarce. In mammalian cells (guinea pig and rat liver) it was found that itaconate is converted to itaconyl-CoA (Adler et al., 1957) and is further processed via

citramalyl-CoA (Wang et al., 1961) to pyruvate and acetyl-CoA. Hereby, it was found that malonate has an inhibitory effect and an addition prevents the degradation of itaconic acid (Adler et al., 1957). The first step of this degradation pathway can be catalyzed by the ubiquitous succinyl-CoA synthetase (Adler et al., 1957; Nagai, 1963; Schürmann et al., 2011). The third step of the pathway is catalyzed by a citramalyl-CoA lyase, where genes from *Chloroflexus aurantiacus* (Friedmann et al., 2007) and *Pseudomonas putida* (Jain, 1996) have been cloned. However, no protein and gene sequence was identified so far, which can catalyze the second step of the degradation pathway, which is an itaconyl-CoA hydratase (Cooper and Kornberg, 1964).

METABOLIC ENGINEERING OF THE ITACONIC ACID PATHWAY IN *A. terreus* AND *A. niger*

The levels of itaconic acid which were reached with *A. terreus* are currently limited to about 85 g/L. Although this is already a substantial amount it cannot be compared with the production of citric acid where titers over 200 g/L are steadily obtained in industrial processes. Transferred to the itaconic acid production a maximal theoretical titer of about 240 g/L should be achievable (Li et al., 2011). This goal could be reached by further breeding of currently existing strains or targeted genetic engineering.

In *A. terreus,* a gene was shown to influence the performance of itaconic acid production, which is a key enzyme of glycolysis. 6-phosphofructo-1-kinase is known to be inhibited by citrate and adenosine triphosphate (ATP). However, a truncated version of the *A. niger pfkA* gene was shown to exhibit a higher citric acid yield due to a reduced inhibition by citrate and ATP (Capuder et al., 2009). This truncated *pfkA* version had also a positive impact on the itaconic acid accumulation when expressed in *A. terreus* (Tevz et al., 2010). Another engineering approach deals with the intracellular oxygen supply. The production of itaconic acid requires continuous aeration and already a short interruption of oxygen decreases the itaconic acid yield. In order to reduce the sensitivity to oxygen a hemoglobin gene from *Vitreoscilla* was expressed in *A. terreus.* Indeed, the expression of this gene leads to an increased itaconic acid production. Furthermore, the strains exhibited a better recovery after the aeration was interrupted (Lin et al., 2004).

There is the possibility that the genetic make-up of *A. terreus* is not efficient enough to support the production of higher titers of organic acids. Therefore, a strategy is to genetically engineer the itaconic acid biosynthesis pathway into another host organism, which is already known to support the production of high titers of organic acids. As already mentioned, *A. niger* is such a candidate. The unique and crucial step in the biosynthesis pathway is the decarboxylation of *cis*-aconitic acid toward itaconic acid. When the *cadA* gene (Kanamasa et al., 2008) was characterized in *A. terreus* genetic engineering of the pathway into another organism became possible. Li et al. (2011) expressed the *A. terreus cadA* gene in *A. niger* strain AB 1.13. For this purpose, the *cadA* gene was placed under the control of the *A. niger gpdA* promoter, which enables a strong and constitutive expression. An *A. niger* strain which expresses the *cadA* gene alone has the ability to produce about 0.7 g/L itaconic acid. This level is not comparable with current production strains of *A. terreus*, but is a promising starting point for further engineering steps. Further attempts to rise the yield are to express genes like the above mentioned mitochondrial carrier protein together with the *cadA* gene (Jore et al., 2011; van der Straat et al., 2012).

OUTLOOK

Itaconic acid as a renewable organic acid is of growing interest for the chemical industry, because of its potential to replace crude oil based products like acrylic acid. Up to now, the microorganism based processes were improved by classical strain breeding and optimizations of the fermentation strategies and conditions. Especially the knowledge about the biotechnological process including oxygen supply, media compositions, and different bioreactor systems was significantly expanded (Kuenz et al., 2012). Regarding the media composition, it was found that copper ions positively influence the itaconic acid production in a genetically engineered *A. niger* strain (Li et al., 2012). However, it is not understood which biochemical reactions are responsible or involved in such an effect. As already mentioned above, the biochemical reactions and effects of itaconic acid in the production hosts are not fully described. The catabolization pathway of itaconic acid requires further investigations in order to engineer a production host with a disabled degradation pathway. The effect of itaconic acid on other metabolic pathways is also of interest because the understanding of its physiological role can prevent undesired side effects (toxicity, health risk, pathway inhibition) and increase the safety of its use. Furthermore, it can be an interesting target for medical research because in mammalian cells it was detected in a metastatic tumor cell line (Strelko et al., 2011). Further knowledge about its role as an enzyme inhibitor can help to develop less-resistant enzyme varieties like in the case of the phosphofructokinase 2. Another target for further engineering is the CadA enzyme, which is described as an unstable protein. Prolonging its *in vivo* stability can help to increase the efficiency of existing production hosts. Also the genetic regulation of the itaconic acid pathway in *A. terreus* requires a profound analysis. Li et al. (2011) have shown that genes involved in the biosynthesis pathway (*cadA*) can be identified by transcriptomic approaches. However, nothing is known so far about the regulatory mechanisms leading to the expression of those genes.

The investigations on the molecular principles of itaconic acid synthesis revealed that *cis*-aconitic acid decarboxylase is the dedicated step in its biosynthesis in *A. terreus.* Genetic engineering of this enzymatic step also renders other microbial hosts like *A. niger* to producers of itaconic acid.

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The dual role of *Candida glabrata* drug:H^+ antiporter CgAqr1 (ORF *CAGL0J09944g*) in antifungal drug and acetic acid resistance

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Opportunistic *Candida* species often have to cope with inhibitory concentrations of acetic acid, in the acidic environment of the vaginal mucosa. Given that the ability of these yeast species to tolerate stress induced by weak acids and antifungal drugs appears to be a key factor in their persistence and virulence, it is crucial to understand the underlying mechanisms. In this study, the drug:H^+ antiporter CgAqr1 (ORF *CAGL0J09944g*), from *Candida glabrata*, was identified as a determinant of resistance to acetic acid, and also to the antifungal agents flucytosine and, less significantly, clotrimazole. These antifungals were found to act synergistically with acetic acid against this pathogen. The action of CgAqr1 in this phenomenon was analyzed. Using a green fluorescent protein fusion, CgAqr1 was found to localize to the plasma membrane and to membrane vesicles when expressed in *C. glabrata* or, heterologously, in *Saccharomyces cerevisiae*. Given its ability to complement the susceptibility phenotype of its *S. cerevisiae* homolog, ScAqr1, CgAqr1 was proposed to play a similar role in mediating the extrusion of chemical compounds. Significantly, the expression of this gene was found to reduce the intracellular accumulation of ^{3}H-flucytosine and, to a moderate extent, of ^{3}H-clotrimazole, consistent with a direct role in antifungal drug efflux. Interestingly, no effect of *CgAQR1* deletion could be found on the intracellular accumulation of ^{14}C-acetic acid, suggesting that its role in acetic acid resistance may be indirect, presumably through the transport of a still unidentified physiological substrate. Although neither of the tested chemicals induces changes in *CgAQR1* expression, pre-exposure to flucytosine or clotrimazole was found to make *C. glabrata* cells more sensitive to acetic acid stress. Results from this study show that CgAqr1 is an antifungal drug resistance determinant and raise the hypothesis that it may play a role in *C. glabrata* persistent colonization and multidrug resistance.

Keywords: *Candida glabrata*, multidrug resistance, drug:H^+ antiporters, acetic acid, flucytosine

INTRODUCTION

Infections caused by *Candida* species are a problem of increasing clinical significance. *Candida glabrata* infections rank second in frequency, immediately after those caused by *C. albicans* (Jarvis, 1995). Although many *Candida* species can be found in the gastrointestinal and genital tract of healthy individuals as innocuous commensals, in immunocompromised hosts they are able to cause skin infections, which may in term lead to invasive infections. In any case, to sense and adapt to different niches within the host environment is essential for their survival and persistence, both as commensals and infection agents.

One of the factors that vary the most within *Candida* colonization sites is pH. Indeed, *Candida* species appear to be suited to thrive in a pH range varying from more than 7.0, as found in the bloodstream, to nearly 4.0, exhibited by the vulvovaginal mucosa. Variation in host niche pH has been seen to affect drug resistance, but the underlying molecular mechanisms are still unclear. Some clues on the mechanisms of the response to pH changes were identified in *C. albicans*. For example, in *C. albicans*, the Rim101 pathway has been shown to be activated under alkaline pH, such as that met during systemic candidiasis (pH 7.4). Rim101 activates the transcription of the *PHR1* gene and represses that of the *PHR2* gene, encoding homologous cell wall β-glycosidases. While Phr1 is required for virulence at alkaline pH, Phr2 is required for virulence at acidic pH, such as that met during vaginal infection (pH 4.0; reviewed in Davis, 2009; Selvig and Alspaugh, 2011). However, little is known on the mechanisms of response and resistance to acidification caused by, or occurring in the presence of weak organic acids, whose concentration reaches quite high values in some colonization or infection sites. Indeed, the concentration of lactic or acetic acid, can reach up to 125 mM in the vaginal tract, particularly under bacterial vaginosis (Chaudry et al., 2004). This is an important issue since the inhibitory effect exerted by weak acids, that dissociate directly in the cytosol leading to intracellular

acidification, is much stronger than that exerted by low extracellular pH *per se* (Mira et al., 2010). Furthermore, the presence of inhibitory or close-to-inhibitory concentrations of these weak acids is likely to interfere with the action of antifungal therapy and, presumably, could also play a role in the induction of multidrug resistance acquisition. For example, fluconazole has been shown to act synergistically with acetic acid in its antifungal action against *C. albicans*. Indeed, its fungicidal activity in vaginal fungal infections appears to be due specifically to the natural existence of weak organic acids in this niche (Moosa et al., 2004).

Within the context of factors conferring resistance to weak acids and drugs, it is interesting to point out that some of the multidrug resistance efflux pumps from the major facilitator superfamily (MFS) characterized in *S. cerevisiae* play a role in weak acid stress resistance (Sá-Correia et al., 2009; Mira et al., 2010). This is the case of the drug:H^+ antiporters Aqr1 (Tenreiro et al., 2002), Azr1 (Tenreiro et al., 2000), Tpo2, and Tpo3 (Fernandes et al., 2005), which have been shown to confer resistance to short chain monocarboxylic acids such as acetic and propionic acids. Among these, Aqr1 stands out as conferring resistance to weak acids, but also to chemical stress inducers such as the antimalarial/antiarrhythmic drug quinidine, the cationic dye crystal violet, or, less clearly, the antifungal drug ketoconazole (Tenreiro et al., 2002). ScAqr1 was further seen to be involved in the excretion of amino acids, particularly homoserine, threonine, alanine, aspartate, and glutamate (Velasco et al., 2004).

This paper describes the functional analysis of the *C. glabrata CgAQR1* gene (ORF *CAGL0J09944g*), sharing a high degree of homology with *S. cerevisiae AQR1* gene, with emphasis on its dual role in acetic acid and antifungal drug resistance. The possible synergy of acetic acid and the antifungal drugs to which CgAqr1 confers resistance to and the ability of acetic acid to induce cross-resistance against antifungal drugs was examined. The sub-cellular localization of this transporter was assessed in *C. glabrata* and its action in reducing the intracellular accumulation of ^{3}H-flucytosine, ^{3}H-clotrimazole, and ^{14}C-acetic acid, in *C. glabrata* cells was evaluated. This study provides an insight into the cross-talk between weak acid and antifungal drug action and resistance, as mediated by CgAqr1, with expected impact in the persistence and multidrug resistance phenotypes exhibited by *C. glabrata* within acidic infection sites in the human host.

MATERIALS AND METHODS

STRAINS, PLASMIDS, AND GROWTH MEDIA

Saccharomyces cerevisiae strain BY4741 (*MAT*a, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0) and the derived single deletion mutant BY4741_Δ*aqr1* were obtained from the Euroscarf collection. The CBS138 *C. glabrata* strain, whose genome sequence was released in 2004, and KUE100 (Ueno et al., 2007) were used in this study. *C. glabrata* strain L5U1 (cgura3Δ0, cgleu2Δ0) was kindly provided by John Bennett (Chen et al., 2007), from the National Institute of Allergy and Infectious Diseases, NIH, Bethesda, USA. The plasmid pGREG576 was obtained from the Drag&Drop collection (Jansen et al., 2005).

Cells were batch-cultured at 30°C, with orbital agitation (250 rpm) in yeast extract peptone dextrose (YPD) growth media, with the following composition: 20 g glucose (Merck), 20 g yeast extract (Difco), and 10 g peptone (Difco). For some of the experiments, minimal medium was used, resulting from different amino acid supplementation of the basal medium (BM) with the following composition (per liter): 1.7 g yeast nitrogen base without amino acids or NH_4^+ (Difco), 20 g glucose (Merck), and 2.65 g $(NH_4)_2SO4$ (Merck). *S. cerevisiae* wild-type and derived BY4741 strains were grown in MM4 medium that resulted from BM supplementation with 20 mg/1 methionine, 20 mg/1 histidine, 60 mg/1 leucine, and 20 mg/1 uracil (all from Sigma). *C. glabrata* strains derived from CBS138 and KUE100 or L5U1 were cultured in BM medium without supplementation or with the supplementation of 20 mg/l uracil and 60 mg/l leucine, respectively. To maintain selective pressure over the recombinant strains, the addition of uracil to this medium was only carried out to grow the host yeast cells. Agarized solid media contained, besides the above-indicated ingredients, 20 g/l agar (Iberagar).

CLONING OF THE *C. glabrata* CgAQR1 GENE (ORF *CAGL0J09944g*)

The pGREG576 plasmid from the Drag&Drop collection (Jansen et al., 2005) was used to clone and express the *C. glabrata* ORF *CAGL0J09944g* in *S. cerevisiae*, as described before for other heterologous genes (Cabrito et al., 2009). pGREG576 was acquired from Euroscarf and contains a galactose inducible promoter (*GAL1*), the yeast selectable marker *URA3* and the *GFP* gene, encoding a green fluorescent protein (GFPS65T), which allows monitoring of the expression and sub-cellular localization of the cloned fusion protein. *CAGL0J09944g* DNA was generated by PCR, using genomic DNA extracted from the sequenced CBS138 *C. glabrata* strain, and the following specific primers: 5′-GAATTCGATATCAAGCTTATCGATACCGTCGACA*ATGGTGGAAAGTGGTCCAC*-3′ and 5′-GCGTGACATAACTAATTACATGACTCGAGGTCGAC*CGTTATCATACTTTTTCTTCAG*-3′. The designed primers contain, besides a region with homology to the first 19 and the last 22 nucleotides of the *CAGL0J09944g* coding region (italic), nucleotide sequences with homology to the cloning site flanking regions of the pGREG576 vector (underlined). The amplified fragment was co-transformed into the parental *S. cerevisiae* strain BY4741 with the pGREG576 vector, previously cut with the restriction enzyme *Sal*I, to obtain the pGREG576_*CgAQR1* plasmid. Since the *GAL1* promoter only allows a slight expression of downstream genes in *C. glabrata*, to visualize by fluorescence microscopy the sub-cellular localization of the *CgAQR1* gene in *C. glabrata*, a new construct was obtained. The *GAL1* promoter present in the pGREG576_*CgAQR1* plasmid was replaced by the copper-inducible *MTI C. glabrata* promoter (Willins et al., 2002), giving rise to the pGREG576_MTI_*CgAQR1* plasmid. The *MTI* promoter DNA was generated by PCR, using genomic DNA extracted from the sequenced CBS138 *C. glabrata* strain, and the following specific primers: 5′-TTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTC*TGTACGACACGCATCATGTGGCAATC*-3′ and 5′-GAAAAGTTCTTCTCCTTTACTCATACTAGTGCGGC*TGTGTTTGTTTTTGTATGTGTTTGTTG*-3′. The designed primers contain, besides a region with homology to the first and last 19 nucleotides of the first 1000 bp of the *MTI* promoter region (italic), nucleotide sequences with homology to the cloning site flanking regions of the pGREG576 vector (underlined). The amplified fragment

was co-transformed into the parental strain BY4741 with the pGREG576_*CgAQR1* plasmid, previously cut with *Sac*I and *Not*I restriction enzymes to remove the *GAL1* promoter, to generate the pGREG576_*MTI_CgAQR1* plasmid. The recombinant plasmids pGREG576_*CgAQR1* and pGREG576_*MTI_CgAQR1* were obtained through homologous recombination in *S. cerevisiae* and verified by DNA sequencing.

DISRUPTION OF THE *C. glabrata* CgAQR1 GENE (ORF *CAGL0J09944g*)

The deletion of the *CgAQR1* gene was carried out in the parental strain KUE100, using the method described by Ueno et al. (2011). The target gene *CAGL0J09944g* (*CgAQR1*) was replaced by a DNA cassette including the CgHIS3 gene, through homologous recombination. The replacement cassette was prepared by PCR using the following primers: 5′-CGTGATCAGCGGCCCGTTATTATTATAGTTTCTTATCTTTTTTTCGTGATGTCCAAAGTTGCCATGTAAA-3′ and 5′-CCAGCCTCACGATGTGATAACGAAACGAAACTCAAAATTACCCAAAATTACCCACAATCAAAACCTAATA-3′. The pHIS906 plasmid including CgHIS3 was used as a template and transformation was performed as described previously (Ueno et al., 2007). Recombination locus and gene deletion were verified by PCR using the following pair of primers: 5′-CTCGTCGTCAGAGTCGTAGT-3′ and 5′-AGAAAACCAGCCTCACGATG-3′.

SUSCEPTIBILITY ASSAYS IN *C. glabrata*

The susceptibility of the parental strain KUE100 toward toxic concentrations of the selected drugs and acetic acid was compared to that of the deletion mutant KUE100_*Δcgaqr1* by spot assays or cultivation in liquid growth medium. The ability of *CgAQR1* gene expression to increase wild-type resistance to the tested chemical stresses was also examined through spot assays in the URA3$^-$ strain L5U1, using the pGREG576_*CgAQR1* centromeric plasmid.

KUE100 *C. glabrata* cell suspensions used to inoculate the agar plates were mid-exponential cells grown in basal BM medium until culture $OD_{600\ nm} = 0.4 \pm 0.02$ was reached and then diluted in sterile water to obtain suspensions with $OD_{600\ nm} = 0.05 \pm 0.005$. These cell suspensions and subsequent dilutions (1:5; 1:25) were applied as 4 μl spots onto the surface of agarized BM medium, supplemented with adequate chemical stress concentrations. The tested drugs included the following compounds, used in the specified concentration ranges: the azole antifungal drugs fluconazole (10–200 mg/l), ketoconazole (10–50 mg/l), clotrimazole (1–20 mg/l), tioconazole (0.2–1 mg/l), and miconazole (0.2–1 mg/l), the polyene antifungal drug amphotericin B (0.1–0.5 mg/l), the fluoropyrimidine 5-flucytosine (0.02–5 mg/l), and the antimalarial/antiarrhythmic drug quinidine (3–9 mg/ml; all from Sigma). L5U1 *C. glabrata* cell suspensions used to inoculate the agar plates were mid-exponential cells grown in basal BM medium, containing 0.5% glucose and 0.1% galactose, without uracil when harboring the pGREG576-derived plasmids, until culture $OD_{600\ nm} = 0.4 \pm 0.02$ was reached and then diluted in sterile water to obtain suspensions with $OD_{600\ nm} = 0.05 \pm 0.005$. These cell suspensions and subsequent dilutions (1:5; 1:25) were applied as 4 μl spots onto the surface of agarized BM medium without uracil, containing 0.1% glucose and 1% galactose, supplemented with adequate chemical stress concentrations, within the above described ranges.

KUE100 *C. glabrata* cell suspensions used for susceptibility assays in liquid medium were grown to mid-exponential phase ($OD_{600\ nm} = 0.4 \pm 0.02$) in basal BM medium, harvested by filtration and re-suspended in fresh BM medium, supplemented or not with the specified concentrations of acetic acid, clotrimazole, or 5-flucytosine, with an initial $OD_{600\ nm} = 0.05 \pm 0.005$. Growth, taking place in Erlenmeyer flasks, at 37°C, 250 rpm, was followed by measuring the optical density of the cell suspension at 600 nm.

SUSCEPTIBILITY ASSAYS IN *S. cerevisiae*

The susceptibility of the parental strain BY4741 toward toxic concentrations of the selected drugs and acetic acid was compared to that of the deletion mutant BY4741_*Δaqr1* by spot assays. The ability of *CgAQR1* gene expression to increase wild-type resistance to the tested chemical stresses and to complement the susceptibility phenotype exhibited by the BY4741_*Δaqr1* single deletion mutants was also examined, using the pGREG576_*CgAQR1* centromeric plasmid in which *CgAQR1* is expressed under the *GAL1* promoter.

Saccharomyces cerevisiae cell suspensions used to inoculate the agar plates were mid-exponential cells grown in basal MM4-U medium, containing 0.5% glucose and 0.1% galactose, until culture $OD_{600\ nm} = 0.4 \pm 0.02$ was reached and then diluted in sterile water to obtain suspensions with $OD_{600\ nm} = 0.05 \pm 0.005$. These cell suspensions and subsequent dilutions (1:5; 1:25) were applied as 4 μl spots onto the surface of agarized MM4-U medium, containing 0.1% glucose and 1% galactose, supplemented with adequate chemical stress concentrations. The tested drugs and other xenobiotics included the following compounds, used in the specified concentration ranges: the azole antifungal drugs fluconazole (50–200 mg/l), itraconazole (40–80 mg/l), ketoconazole (10–50 mg/l), clotrimazole (1–20 mg/l), tioconazole (0.05–0.2 mg/l), and miconazole (0.05–0.2 mg/l), the polyene antifungal drug amphotericin B (0.05–0.5 mg/l), the fluoropyrimidine 5-flucytosine (0.02–5 mg/l), and the weak organic monocarboxylic acid acetic acid (30–60 mM) (all from Sigma).

CgAqr1 SUB-CELLULAR LOCALIZATION ASSESSMENT

The sub-cellular localization of the CgAqr1 protein was determined based on the observation of BY4741 *S. cerevisiae* or L5U1 *C. glabrata* cells transformed with the pGREG576-*CgAQR1* or pGREG576-*MTI-CgAQR1* plasmids, respectively. These cells express the CgAqr1_GFP fusion protein, whose localization may be determined using fluorescence microscopy. *S. cerevisiae* cell suspensions were prepared by cultivation in MM4-U medium, containing 0.5% glucose and 0.1% galactose, at 30°C, with orbital shaking (250 rev/min), until a standard culture $OD_{600\ nm}$ (Optical Density at 600 nm) $= 0.4 \pm 0.04$ was reached. At this point cells were transferred to the same medium containing 0.1% glucose and 1% galactose, to induce protein expression. *C. glabrata* cell suspensions were prepared in BM-U medium, until a standard culture $OD_{600\ nm} = 0.4 \pm 0.04$ was reached, and transferred to the same medium supplemented with 30 μM $CuSO_4$ (Sigma), to

induce protein over-expression. After 5 h of incubation, the distribution of CgAqr1_GFP fusion protein in *S. cerevisiae* or in *C. glabrata* living cells was detected by fluorescence microscopy in a Zeiss Axioplan microscope (Carl Zeiss MicroImaging), using excitation and emission wavelength of 395 and 509 nm, respectively. Fluorescence images were captured using a cooled CCD camera (Cool SNAPFX, Roper Scientific Photometrics).

TRANSPORT ASSAYS USING RADIOLABELED COMPOUNDS

[^{3}H]-flucytosine, [^{3}H]-clotrimazole, and [^{14}C]-acetate accumulation assays were carried out as described before (Vargas et al., 2004). To estimate the accumulation of each radiolabeled compound (intracellular/extracellular) in yeast cells, the parental strain KUE100 and the mutant strain KUE100_*Δcgaqr1* were grown in BM medium till mid-exponential phase and harvested by filtration. Cells were washed and re-suspended in BM medium, to obtain dense cell suspensions [$OD_{600\ nm} = 5.0 \pm 0.1$, equivalent to approximately 2.2 mg (dry weight)/ml]. After 5 min incubation at 30°C, with agitation (150 rpm), the radiolabeled compound was added to the cell suspensions [1 μM of [^{3}H]-flucytosine (American Radiolabeled Chemicals; 1 mCi/ml) and 3 mg/l of unlabeled flucytosine; or 0.1 μM of [^{3}H]-clotrimazole (American Radiolabeled Chemicals; 1 mCi/ml) and 75 mg/l of unlabeled clotrimazole; 3.5 μM of [^{14}C]-acetic acid (American Radiolabeled Chemicals; 0.1 mCi/ml) and 100 mM of unlabeled acetic acid]. The accumulation of each radiolabeled compound was followed, in individual assays, for an additional period of 30 min until equilibrium was reached. In all cases, the intracellular accumulation of the radiolabeled compound was followed by filtering 200 μl of cell suspension, at adequate time intervals, through pre-wetted glass microfiber filters (Whatman GF/C). The filters were washed with ice-cold TM buffer and the radioactivity measured in a Beckman LS 5000 TD scintillation counter. Extracellular concentration of the radiolabeled compound was estimated, by radioactivity assessment of 50 μl of the supernatant.

Non-specific adsorption of each radiolabeled compound to the filters and to the cells (less than 5% of the total radioactivity) was assessed and taken into consideration. To calculate the intracellular concentration of each radiolabeled compound, the internal cell volume (V_i) of the exponential cells, grown in the absence of drug and used for accumulation assays, was considered constant and equal to 2.5 μl/mg dry weight (Rosa and Sá-Correia, 1996).

CgAqr1 EXPRESSION MEASUREMENTS

The levels of *CgAQR1* transcripts in *C. glabrata* cells were assessed by quantitative real-time PCR. Total RNA samples were obtained from cell suspensions harvested in control conditions (mid-exponential phase cells in the absence of drugs) or upon 1 h of exposure to 75 mg/l clotrimazole or 3.5 mg/l flucytosine or 60 mM acetic acid. Synthesis of cDNA for real-time RT-PCR experiments, from total RNA samples, was performed using the Multiscribe™ reverse transcriptase kit (Applied Biosystems) and the 7500 RT-PCR Thermal Cycler Block (Applied Biosystems), following the manufacturer's instructions. The quantity of cDNA for the following reactions was kept around 10 ng. The subsequent RT-PCR step was carried out using SYBR® Green reagents. Primers for the amplification of the *CgAQR1* and *CgACT1* cDNA were designed using Primer Express Software (Applied Biosystems) and are 5′-GCTGATAAGTTCGGCCGTAGA-3′ and 5′-AATGGAGGCAACCACGTAGATC-3′ and 5′-AGAGCCGTCT TCCCTTCCAT-3′ and 5′-TTGACCCATACCGACCATGA-3′, respectively. The RT-PCR reaction was carried out using a thermal cycler block (7500 Real-Time PCR System – Applied Biosystems). Default parameters established by the manufacturer were used and fluorescence detected by the instrument and registered in an amplification plot (7500 System SDS Software – Applied Biosystems). The *CgACT1* mRNA level was used as an internal control. The relative values obtained for the wild-type strain in control conditions were set as 1 and the remaining values are presented relative to that control. To avoid false positive signals, the absence of non-specific amplification with the chosen primers was confirmed by the generation of a dissociation curve for each pair of primers.

RESULTS

CgAqr1 EXPRESSION CONFERS RESISTANCE TO ACETIC ACID

Given the previously observed effect of *S. cerevisiae AQR1* gene in weak acid stress tolerance (Tenreiro et al., 2002), the action of CgAqr1 in acetic acid tolerance was inspected. The deletion of *CgAQR1* gene in *C. glabrata* clearly decreases its tolerance to an inhibitory concentration of acetic acid (**Figure 1**). This is visible both in spot assays (**Figure 1A**) and in liquid medium cultivation (**Figure 1B**). Sudden exposure of un-adapted wild-type *C. glabrata* cells to 60 mM acetic acid leads to 5 h of lag-phase followed by growth resumption with reduced kinetics, while cells devoid of CgAqr1 enter a lag-phase that lasts for around 18 h, before growth resumption. Apparently, the absence of the Aqr1 gene affects only slightly the growth rate at which cells are able to reassume exponential growth in the presence of acetic acid, suggesting that its role is mainly played during the adaptation to this stress (**Table 1**). The introduction of a recombinant plasmid expressing *CgAqr1* further increases *C. glabrata* natural resistance toward acetic acid, when compared to the same strain harboring the corresponding cloning vector (**Figure 1C**), reinforcing the finding that *CgAqr1* is a determinant of acetic acid resistance in *C. glabrata*.

CgAqr1 EXPRESSION CONFERS RESISTANCE TO FLUCYTOSINE AND AZOLE ANTIFUNGAL DRUGS

The deletion of the *CgAqr1* gene in *C. glabrata* was found, based on spot assays, to increase the susceptibility of this pathogen against the antifungal fluoropyrimidine analog flucytosine, and the imidazole antifungal drugs miconazole, clotrimazole, and tioconazole (**Figure 2A**). The introduction of a recombinant plasmid expressing *CgAqr1* increases *C. glabrata* natural resistance toward flucytosine, but only very mildly against clotrimazole and tioconazole, when compared to the same strain harboring the corresponding cloning vector (**Figure 2B**), reinforcing the finding that *CgAqr1* is a strong determinant of flucytosine resistance in *C. glabrata*. No effect of CgAqr1 expression could be clearly detected in *C. glabrata* susceptibility to fluconazole, itraconazole, or amphotericin B. The comparison of the growth curves of wild-type and derived *Δcgaqr1* cells in liquid medium show that the deletion of CgAqr1 clearly impairs *C. glabrata* growth in the presence of clotrimazole and, particularly, flucytosine (**Figure 3**). Indeed, although the growth curves of both strains are nearly

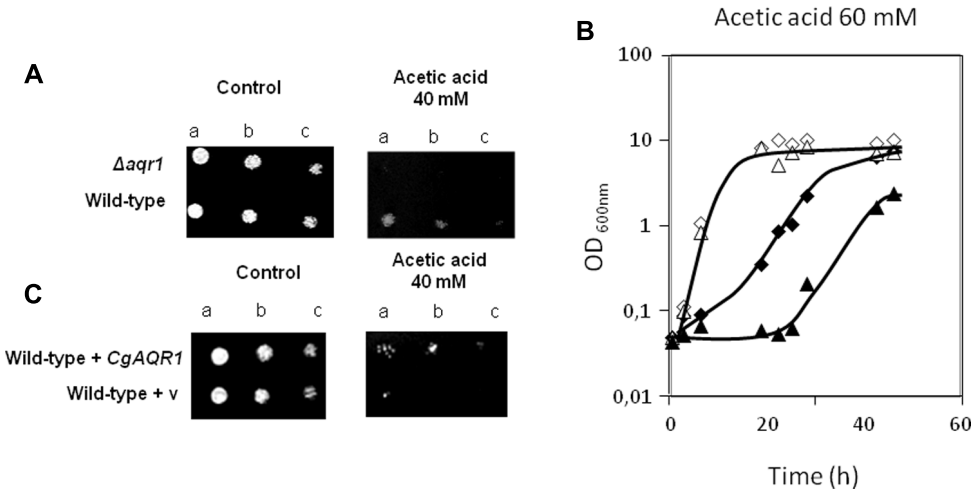

FIGURE 1 | (A) Comparison of the susceptibility to inhibitory concentrations of acetic acid, at the indicated concentration, of the *C. glabrata* KUE100 and KUE100_*Δcgaqr*1 strains, in BM agar plates by spot assays.**(B)**Comparison of growth curves of the *C. glabrata* KUE100 (◇,◆) and KUE100_*Δcgaqr1* (Δ,▲) strains, in liquid BM medium, supplemented with 0 mM (◇,Δ) or 60 mM (◆,▲) of acetic acid.**(C)**Comparison of the susceptibility to inhibitory concentrations of acetic acid, at the indicated concentration, of the *C. glabrata* L5U1 strain, harboring the pGREG576 cloning vector (v) or the pGREG576_*CgAqr1* plasmid in BM agar plates, without uracil, by spot assays. The inocula were prepared as described in Section "Materials and Methods." Cell suspensions used to prepare the spots were 1:5 (b) and 1:25 (c) dilutions of the cell suspension used in (a). The displayed images and growth curves are representative of at least three independent experiments.

indistinguishable in control conditions, upon sudden exposure to the presence of 3 mg/ml of flucytosine the wild-type cells experience a period of around 18 h of lag-phase, followed by exponential growth with a reduced rate, while the *Δcgaqr1* deletion mutant cells enter a period of lag-phase that last for around 35 h prior to growth resumption with even more inhibited growth kinetics (**Table 1**; **Figure 3A**). A less striking, but still clear, effect of CgAqr1 deletion can also be seen in the presence of 50 mg/ml of clotrimazole. *Δcgaqr1* deletion mutant cells exhibit a period of lag-phase of around 18 h prior to growth resumption, while the effect of this concentration of clotrimazole in wild-type cells appears to be felt mostly at the level of growth rate inhibition (**Table 1**; **Figure 3B**).

Using *S. cerevisiae* as a heterologous expression system, the effect of *cgaqr1* expression in yeast resistance to antifungal drugs was further tested, in order to verify whether or not *cgaqr1* is able to functionally complement its *S. cerevisiae* homolog. The deletion of the *S. cerevisiae AQR1* gene was found to increase the susceptibility toward flucytosine, clotrimazole and, as observed before (Tenreiro et al., 2002), acetic acid exhibited by the corresponding parental strain (**Figure 4**). When expressed in the *S. cerevisiae Δaqr1* background, the *cgaqr1* gene was able to rescue all the observed susceptibility phenotypes, further confirming its role in flucytosine and imidazole drug resistance (**Figure 4**).

ACETIC ACID ACTS SYNERGISTICALLY WITH CLOTRIMAZOLE AND FLUCYTOSINE

In order to evaluate the eventual effect of acetic acid, present in some of the natural niches of *Candida* infection, in antifungal therapy, the effect of co-exposure of *C. glabrata* cells to acetic acid and to the antifungals clotrimazole and flucytosine was examined. *C. glabrata* wild-type cells were exposed to concentrations of acetic acid, clotrimazole, and flucytosine that, individually, do not affect growth: 45 mM, 30 and 0.2 mg/l, respectively (**Figure 5**). However, when in combination, the same "innocuous" concentrations of acetic acid and clotrimazole or acetic acid and flucytosine, were found to lead to severe growth impairment. Indeed, when grown in the presence of 30 mg/l clotrimazole plus 45 mM of acetic acid, the *C. glabrata* population enters a period of 40 h of lag-phase, followed by a drastically reduced growth rate and reaching lower levels of final biomass when compared to control cells (**Figure 5**). Sudden exposure to 0.2 mg/l flucytosine plus 45 mM of acetic acid leads to an even longer period of lag-phase (50 h), upon

Table 1 | Comparison of the susceptibility to inhibitory concentrations of acetic acid, clotrimazole, or flucytosine of the *C. glabrata* KUE100 and KUE100_*Δcgaqr1* strains, based on the growth parameters lag-phase duration, exponential growth rate, and final biomass.

Growth conditions	Strain	Lag-phase (h)	Growth rate/h	Final biomass ($OD_{600\ nm}$)
Control	Wild-type	nd	1.32 ± 0.08	9.76 ± 0.39
	Δaqr1	nd	1.23 ± 0.09	6.64 ± 0.96
Clotrimazole	Wild-type	6.25 ± 2.71	0.20 ± 0.04	6.06 ± 0.09
50 mg/l	*Δaqr1*	17.96 ± 0.27	0.19 ± 0.01	3.00 ± 0.17
Flucytosine	Wild-type	18.76 ± 0.13	0.30 ± 0.02	9.28 ± 0.51
3 mg/l	*Δaqr1*	35.14 ± 3.60	0.21 ± 0.07	4.06 ± 0.37
Acetic acid	Wild-type	5.31 ± 2.86	0.19 ± 0.02	7.76 ± 0.45
60 mM	*Δaqr1*	18.28 ± 1.08	0.14 ± 0.001	2.36 ± 0.01

Values are the average of at least three independent experiments ± standard deviation. Nd, not detected.

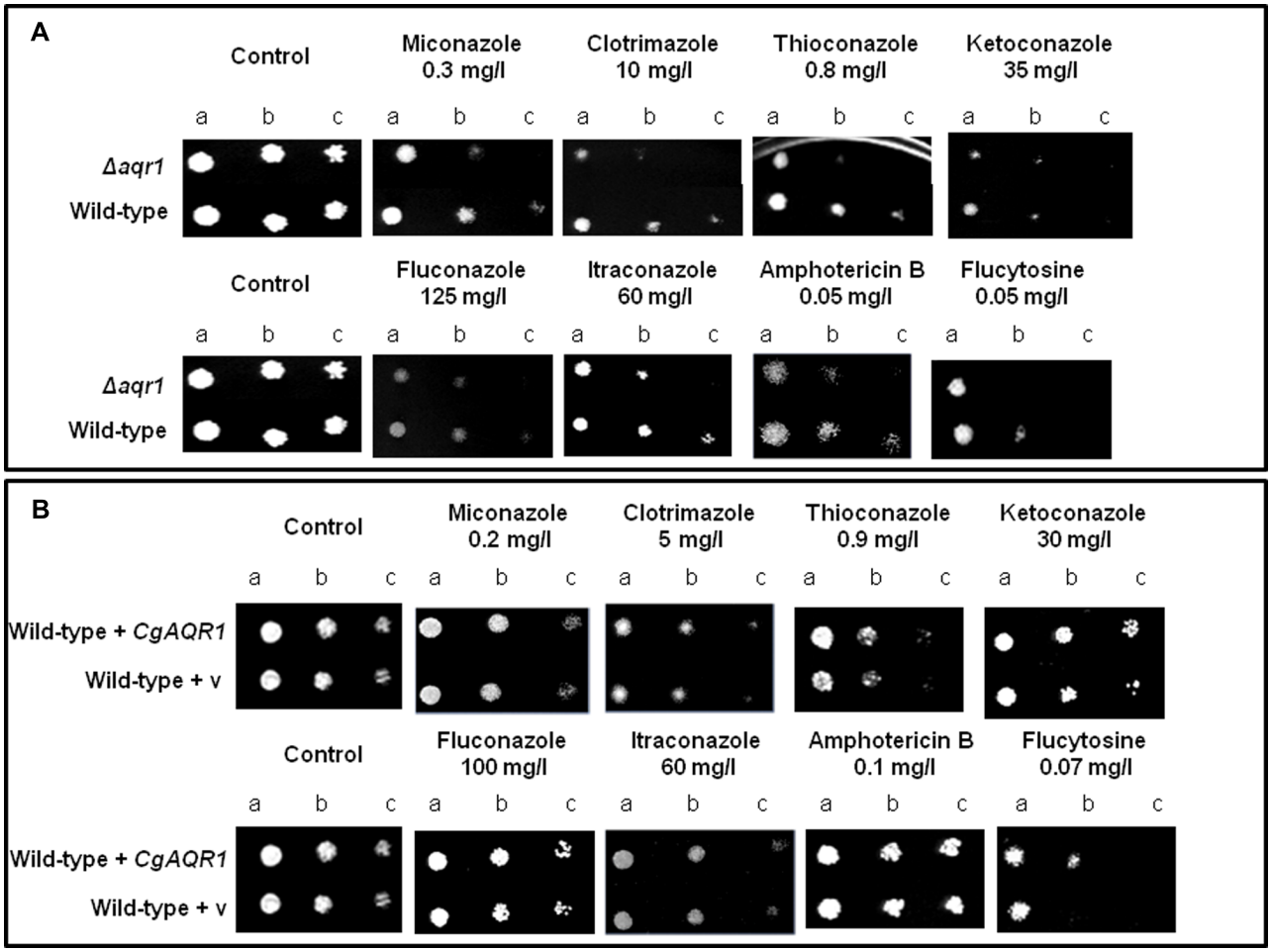

FIGURE 2 | (A) Comparison of the susceptibility to antifungal drugs, at the indicated concentrations, of the *C. glabrata* KUE100 and KUE100_*Δcgaqr1* strains, in BM agar plates by spot assays. **(B)** Comparison of the susceptibility to antifungal drugs, at the indicated concentrations, of the *C. glabrata* L5U1 strain, harboring the pGREG576 cloning vector (v) or the pGREG576_*CgAqr1* plasmid in BM agar plates, without uracil, by spot assays. The inocula were prepared as described in Section "Materials and Methods." Cell suspensions used to prepare the spots were 1:5 (b) and 1:25 (c) dilutions of the cell suspension used in (a). The displayed images are representative of at least three independent experiments.

which growth resumption is achieved (**Figure 5**). It is clear that the inhibitory effect of *C. glabrata* exposure to acetic acid plus flucytosine or acetic acid plus clotrimazole is much stronger than the sum of their individual inhibitory effects, suggesting that acetic acid acts synergistically with flucytosine and clotrimazole. In the absence of CgAqr1, this effect seems to be even more pronounced. Given the fact that flucytosine is used mostly in combination with fluconazole, we further verified experimentally that flucytosine and fluconazole do exhibit a synergistic effect. However, and as expected based on the fact that CgAqr1 does not confer resistance to fluconazole, no increased role of CgAqr1 in the presence of this combination of drugs was observed (results not shown).

CgAqr1 IS LOCALIZED TO THE PLASMA MEMBRANE AND MEMBRANE VESICLES IN *C. glabrata* AND IN *S. cerevisiae*

Candida glabrata cells harboring the pGREG576_*MTI_CgAQR1* plasmid were grown to mid-exponential phase in minimal medium, and then transferred to the same medium containing 30 μM $CuSO_4$, to promote protein expression in moderate controlled levels. At a standard $OD_{600\ nm}$ of 0.5 ± 0.05, obtained after around 5 h of incubation, cells were inspected through fluorescence microscopy. This period of incubation was found to allow detectable protein expression levels, but not a high degree of over-expression that may lead to mis-localization. In *C. glabrata* cells, the CgAqr1_GFP fusion was found to be localized to the cell periphery, and also to a punctuate distribution throughout the cell (**Figure 1A**). Control cells, on the other hand, harboring the pGREG576 cloning vector, displayed a slight and uniform distribution of fluorescence (**Figure 6A**), similar to what can be observed as the host cells auto-fluorescence. Since CgAqr1 is predicted to be an integral membrane protein (Gbelska et al., 2006), these results strongly suggest a plasma membrane and, eventually, membrane vesicle localization, similar to what was observed for its *S. cerevisiae* homolog Aqr1 (Tenreiro et al., 2002; Velasco et al., 2004). *S. cerevisiae* cells harboring the pGREG576_*CgAqr1* plasmid were also tested for the sub-cellular localization of CgAqr1, to verify that

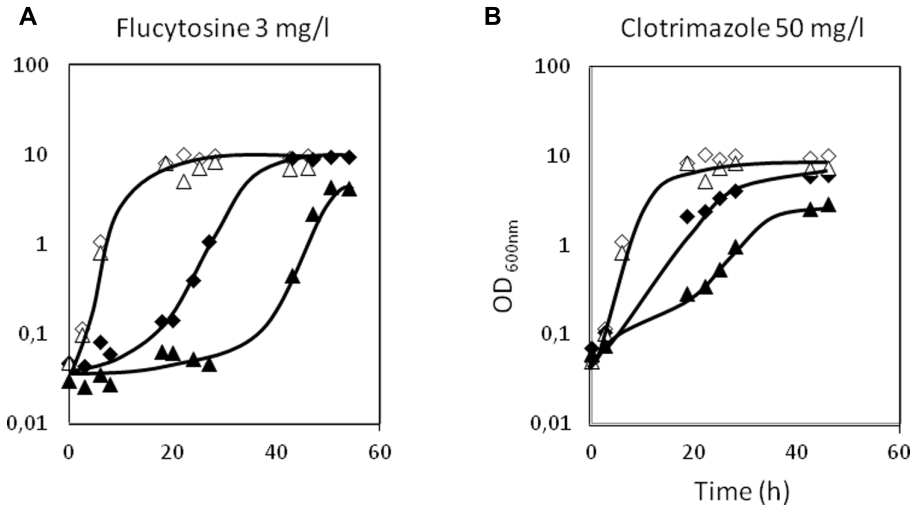

FIGURE 3 | Comparison of the growth curves of *C. glabrata* KUE100 (◇, ♦) and KUE100_*Δcgaqr1* (Δ,▲) strains, in liquid BM medium, in the absence (◇,Δ) or presence of 3 mg/l flucytosine (A) or 50 mg/l clotrimazole (B) (♦,▲). The inocula were prepared as described in Section "Materials and Methods." The displayed growth curves are representative of at least three independent experiments.

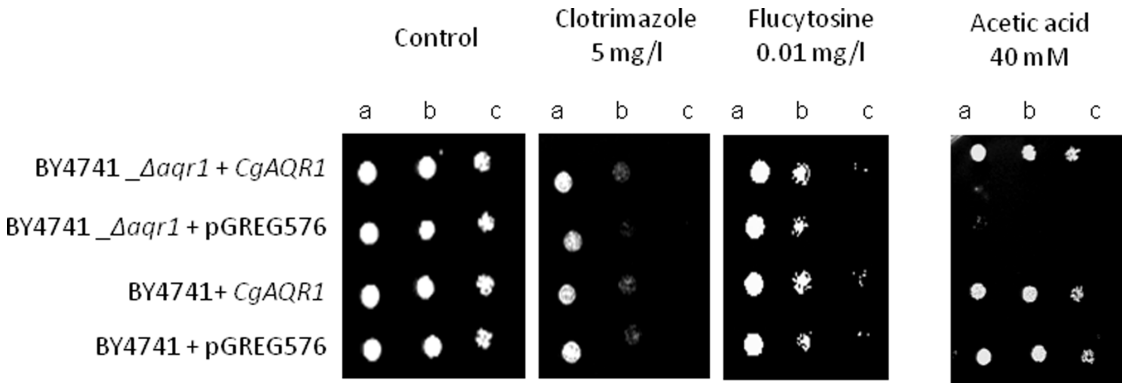

FIGURE 4 | Comparison of the susceptibility to antifungal drugs and acetic, at the indicated concentrations, of the *S. cerevisiae* BY4741 and BY4741_*Δaqr1* strains, harboring the cloning vector pGREG576 (v) or the derived *cgaqr1* expression plasmid pGREG_*cgaqr1*, in MM4 agar plates by spot assays. Cell suspensions used to prepare the spots were 1:5 (b) and 1:25 (c) dilutions of the cell suspension used in (a). The inocula were prepared as described in Section "Materials and Methods." Images are representative of at least three independent experiments.

in these cells, the *C. glabrata* transporter was similarly localized to the plasma membrane and membrane vesicles. Cells were grown to mid-exponential phase in minimal medium containing 0.5% glucose and 0.1% galactose, and then transferred to the same medium containing 0.1% glucose and 1% galactose, to promote protein over-expression. At a standard $OD_{600\ nm}$ of 0.5 ± 0.05, obtained after around 5 h of incubation, cells were inspected through fluorescence microscopy and plasma membrane and membrane vesicle localization was verified (**Figure 6B**).

CgAqr1 PLAYS A ROLE IN REDUCING THE INTRACELLULAR ACCUMULATION OF ^{3}H-flucytosine, BUT NOT OF ^{3}H-CLOTRIMAZOLE OR ^{14}C-ACETIC ACID IN *C. glabrata*

Since the *C. glabrata* gene *CgAqr1*, was found herein to encode a drug resistance transporter of the plasma membrane, and of what appears to be the membrane of exocytic vesicles, and to act as a determinant of resistance to flucytosine, acetic acid, and less significantly, clotrimazole, its possible involvement in reducing the accumulation of these compounds in challenged yeast cells was examined. The accumulation of radiolabeled flucytosine was seen to be four times higher in cells devoid of CgAqr1 than in wild-type cells (**Figure 7A**), correlating with the strong effect of *CgAQR1* deletion in flucytosine resistance (**Figure 3**). The accumulation of [^{3}H]-labeled clotrimazole in non-adapted *C. glabrata* cells suddenly exposed to the presence of 30 mg/l cold clotrimazole was also tested and found to be slightly higher than in cells devoid of CgAqr1 than in parental KUE100 cells (**Figure 7B**), which appears to be consistent with the relatively small difference in growth inhibition exhibited by wild-type and *Δcgaqr1* cells. Surprisingly, no clear difference in ^{14}C-acetic acid accumulation could be found in wild-type and *Δcgaqr1* strains (**Figure 7C**). These results strongly suggests that CgAqr1 activity increases yeast resistance

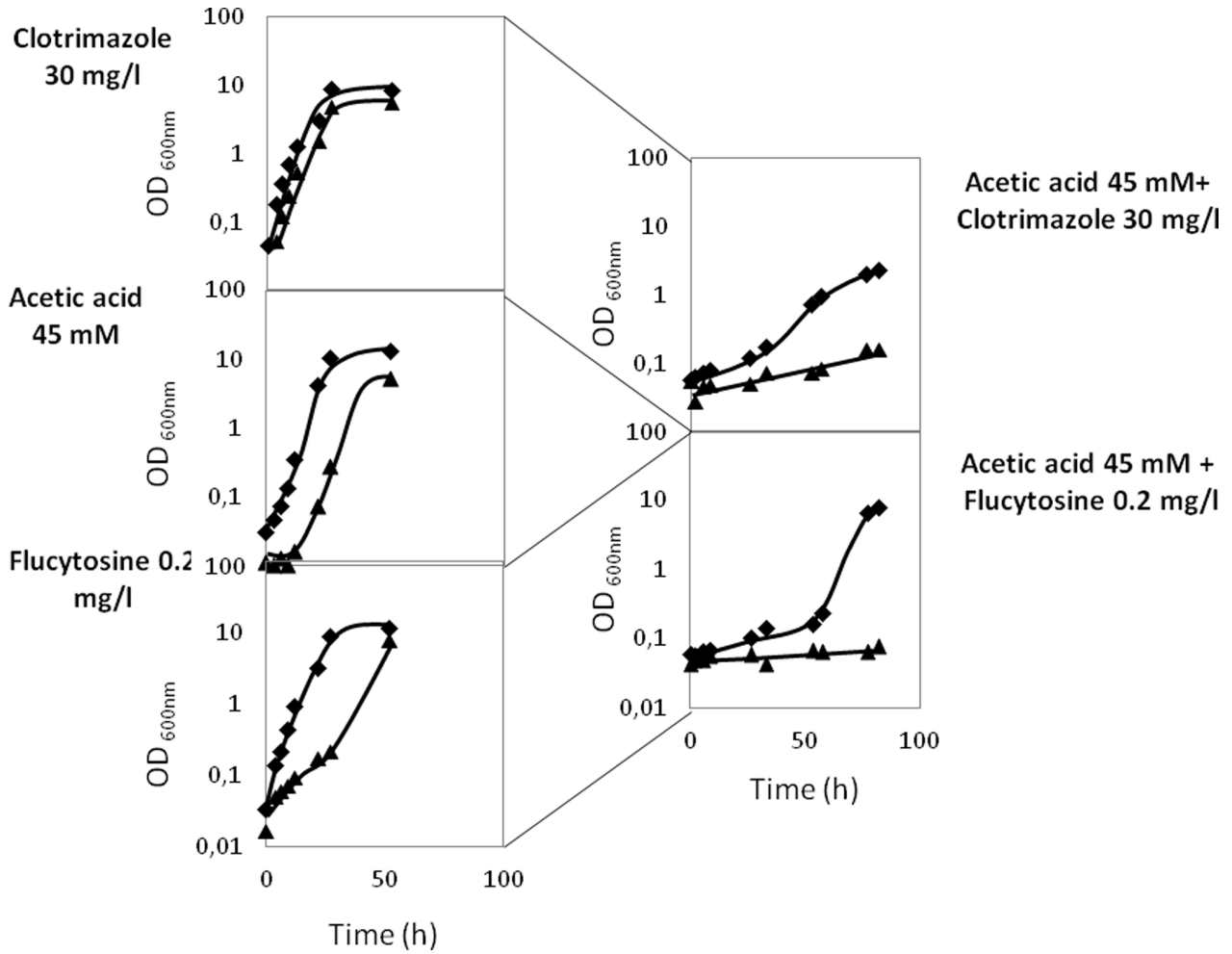

FIGURE 5 | Comparison of the growth curves of *C. glabrata* KUE100 (♦) and KUE100_*Δcgaqr1* (▲) strains, in liquid BM medium, in the presence of clotrimazole, acetic acid, or flucytosine (on the right) or in the simultaneous presence of acetic acid and clotrimazole or acetic acid and flucytosine (on the left), at the indicated concentrations. The inocula were prepared as described in Section "Materials and Methods." The displayed growth curves are representative of at least three independent experiments.

toward flucytosine by reducing its accumulation within yeast cells, presumably by catalyzing the direct extrusion of this antifungal drug, while its action in acetic acid stress tolerance may be indirect.

CgAqr1 TRANSCRIPT LEVELS ARE NOT UP-REGULATED UNDER ACETIC ACID, CLOTRIMAZOLE, OR FLUCYTOSINE STRESS

The effect of *C. glabrata* cell exposure to inhibitory concentrations of acetic acid, clotrimazole or flucytosine, to which CgAqr1 confers resistance, in *CgAqr1* transcription was evaluated. The transcript levels of *CgAqr1* gene were seen to suffer no significant change upon 1 h of exposure of an un-adapted *C. glabrata* population to inhibitory concentrations of acetic acid, clotrimazole or flucytosine (**Figure 8**).

ACETIC ACID DOES NOT PROVIDE CROSS-PROTECTION AGAINST CLOTRIMAZOLE AND FLUCYTOSINE

Given that CgAqr1 provides *C. glabrata* protection against acetic acid, flucytosine, and clotrimazole, the possibility that exposure to each of these stress agents might confer cross-protection against the remaining was hypothesized. To evaluate this possibility, *C. glabrata* cells were pre-exposed to 60 mM of acetic acid, 50 mg/l of clotrimazole, and 0.5 mg/l of flucytosine and cultivated until mid-exponential phase was reached ($OD_{600\ nm} = 1.5 \pm 0.05$). These cells, growing exponentially in the presence of each of these stress agents were then harvested, washed, and exposed to the three growth inhibitors as secondary stress agents. Pre-exposure to acetic acid made *C. glabrata* cells able to growth at maximal exponential rate upon re-exposure to the same concentration of the acid, but did not increase yeast resistance to clotrimazole or flucytosine (**Figure 9**). On the other hand, pre-exposure to clotrimazole appears to have no protective effect against flucytosine or, not even, to re-exposure to clotrimazole itself, and to induce a sensitization against post-exposure to acetic acid (**Figure 9**). Finally, pre-exposure to flucytosine, while making *C. glabrata* cells tolerant to re-exposure to flucytosine did actually make the cells more susceptible to acetic acid and clotrimazole (**Figure 9**).

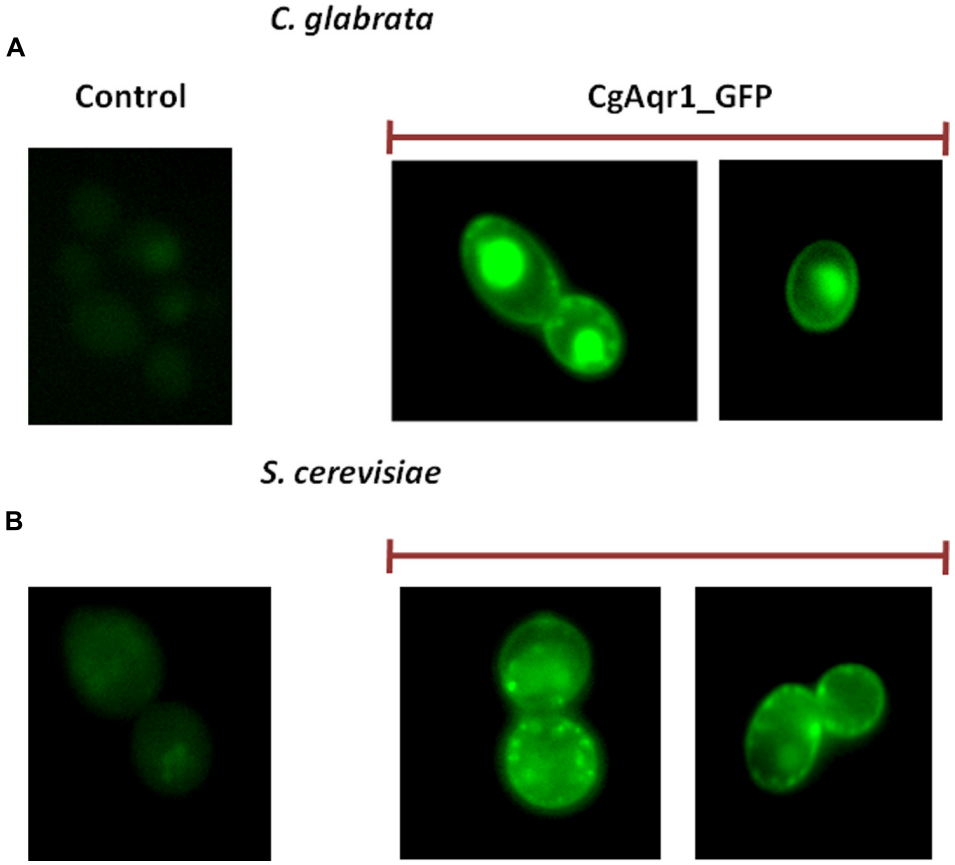

FIGURE 6 | Fluorescence of exponential phase L5U1 *C. glabrata* cells (A) or BY4741 *S. cerevisiae* cells (B), harboring the cloning vector pGREG576 (control) or the pGREG576_*CgAqr1* or pGREG576_MTI_*CgAqr1* plasmids (CgAqr1_GFP), after 5 h of galactose- or copper-induced recombinant protein production, respectively. Results indicate that the CgAqr1-GFP fusion protein localizes to the plasma membrane and membrane vesicles in both *S. cerevisiae* and *C. glabrata* cells.

DISCUSSION

In this study, the functional characterization of the *C. glabrata* CgAqr1 drug:H^+ antiporter was carried out. CgAqr1 was identified as the first of its family to confer resistance in this species to the antifungal drug flucytosine, but also to acetic acid.

To the best of our knowledge, no multidrug resistance transporter had ever been linked to flucytosine resistance. This is quite surprising, considering that it has been known for some time that the acquisition of flucytosine resistance among clinical isolates occurs very fast, which appears to be compatible with the action of drug efflux pumps (Thompson et al., 2009). In most cases, flucytosine resistance has been associated to point mutations or changes in the expression of the genes involved in the uptake (e.g., carried out by cytosine permeases, such as Fcy2) and catabolism (the first step carried out by cytosine deaminase Fcy1) of this antifungal pro-drug (Hope et al., 2004). Significantly, results from this study point out to a direct role of CgAqr1 in flucytosine efflux. Indeed, the increased flucytosine resistance observed in cells expressing the *CgAQR1* gene, when compared to *Δcgaqr1* deletion mutant cells, appears to correlate with the observation that the accumulation of this antifungal drug is twofold higher in the deletion mutant, when compared to the corresponding parental strain. The results displayed herein further highlight the unexplored role of drug efflux pumps in the context of flucytosine resistance.

CgAqr1 was also found to confer, to a lower level, resistance to azole drugs, including the imidazoles miconazole, tioconazole, and clotrimazole, used in the treatment of fungal skin infections and vaginal or oral candidemia. In *C. glabrata*, resistance to azole drugs in clinical isolates has been shown to depend often on the action of the ABC drug efflux pumps encoded by *CgCDR1*, *CgCDR2*, and *CgAUS1* (Cannon et al., 2009). Very recently, the *C. glabrata* CgQdr2 drug:H^+ antiporter was identified as the first of its family to confer imidazole drug resistance, playing a direct role in the extrusion of clotrimazole (Costa et al., 2013). In this study, CgAqr1 is added to the number of characterized *Candida* transporters involved in azole drug resistance. However, the deletion of *CgAQR1* was found to have only a slight effect on ^{3}H-clotrimazole accumulation in *C. glabrata* cells, consistent with the moderate increase in susceptibility registered upon *CgAQR1* deletion.

Based on its high degree of homology and functional similarity to the *S. cerevisiae AQR1* gene, a possible physiological role for CgAqr1 linked to yeast survival in the presence of inhibitory concentrations of acetic acid (Tenreiro et al., 2002) was also

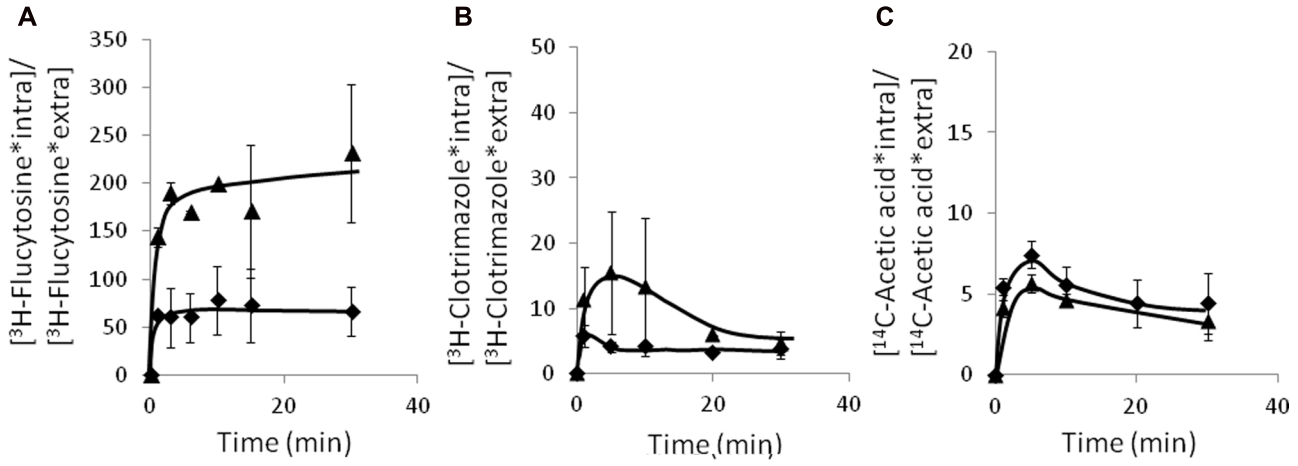

FIGURE 7 | Time course accumulation ratio of [^{3}H]-flucytosine (A), [^{3}H]-clotrimazole (B), and [^{14}C]-acetate (C) in non-adapted cells of KUE100 (♦) or KUE100_*Δcgaqr1* (▲) strains, during cultivation in BM liquid medium in the presence of 3 mg/l, 30 mg/l, or 100 mM of unlabeled flucytosine, clotrimazole, or acetic acid, respectively. The accumulation ratio values are averages of at least three independent experiments. Error bars represent the corresponding standard deviations.

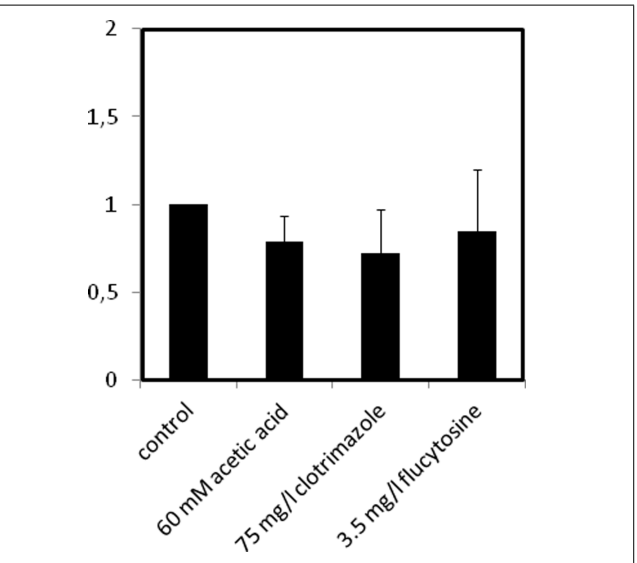

FIGURE 8 | Comparison of the variation of the *CgAQR1* transcript levels in wild-type *C. glabrata* cells before (control) and after 1 h of exposure to acetic acid-, clotrimazole-, or flucytosine-induced stress, at the indicated concentrations. The presented transcript levels were obtained by quantitative RT-PCR, as described in Section "Materials and Methods," and are relative *CgAQR1/CgACT1* mRNA values, considering the value registered in control conditions, equal to 1. The obtained values are the average of at least three independent experiments. Error bars represent the corresponding standard deviations.

inspected. Indeed, CgAqr1 expression did improve *C. glabrata* and *S. cerevisiae* fitness under inhibitory concentrations of this weak carboxylic acid, and complemented the *S. cerevisiae Δaqr1* susceptibility phenotype observed in these conditions. ScAqr1 and CgAqr1 were further found to confer flucytosine resistance in *S. cerevisiae*, CgAqr1 being able to complement the absence of its *S. cerevisiae* counterpart under flucytosine stress. The notion that ScAqr1 and CgAqr1 may have overlapping functions is reinforced by the finding that their sub-cellular localization is also quite similar, while different from that of the remaining members of their family (Sá-Correia et al., 2009). Indeed, similar to what had been registered for ScAqr1 (Tenreiro et al., 2002; Velasco et al., 2004), CgAqr1 was found to be localized to both the plasma membrane and membrane vesicles, being proposed to catalyze the extrusion of its substrates across the plasma membrane or through exocytic vesicles. The possibility that CgAqr1 displays as physiological role the catalysis of acetate excretion was further investigated, but no effect of *CgAQR1* expression on ^{14}C-acetate accumulation could be detected. Significantly, the expression of the *S. cerevisiae* Aqr1 transporter had also been found to have no effect on acetate transport (Tenreiro et al., 2002). Although the exact role of CgAqr1 in acetic acid resistance remains to be perceived, it may be indirectly due to the transport of a still unidentified physiological substrate. Since ScAqr1 was seen to be involved in the excretion of amino acids, particularly homoserine, threonine, alanine, aspartate, and glutamate (Velasco et al., 2004), it is reasonable to hypothesize that a similar trait may be exhibited by its ortholog in *C. glabrata*. Although it appears difficult to foresee how exactly amino acid excretion may contribute to acetic acid and azole drug resistance, the truth is that the molecular mechanisms behind the apparent promiscuity exhibited by MDR transporters of the ABC and MFS superfamilies remains elusive and controversial (Roepe et al., 1996; Prasad et al., 2002; Sá-Correia and Tenreiro, 2002; Paulsen, 2003; Jungwirth and Kuchler, 2006; Sá-Correia et al., 2009). Furthermore, the fact that some of the compounds to which multidrug transporters confer resistance to are not extruded through their direct action has been observed for the majority of the ABC and MFS–MDR transporters characterized in *S. cerevisiae* (Jungwirth and Kuchler, 2006; Sá-Correia et al., 2009), whereas they have been found to be involved in the transport of physiological substrates, including membrane lipids and ions

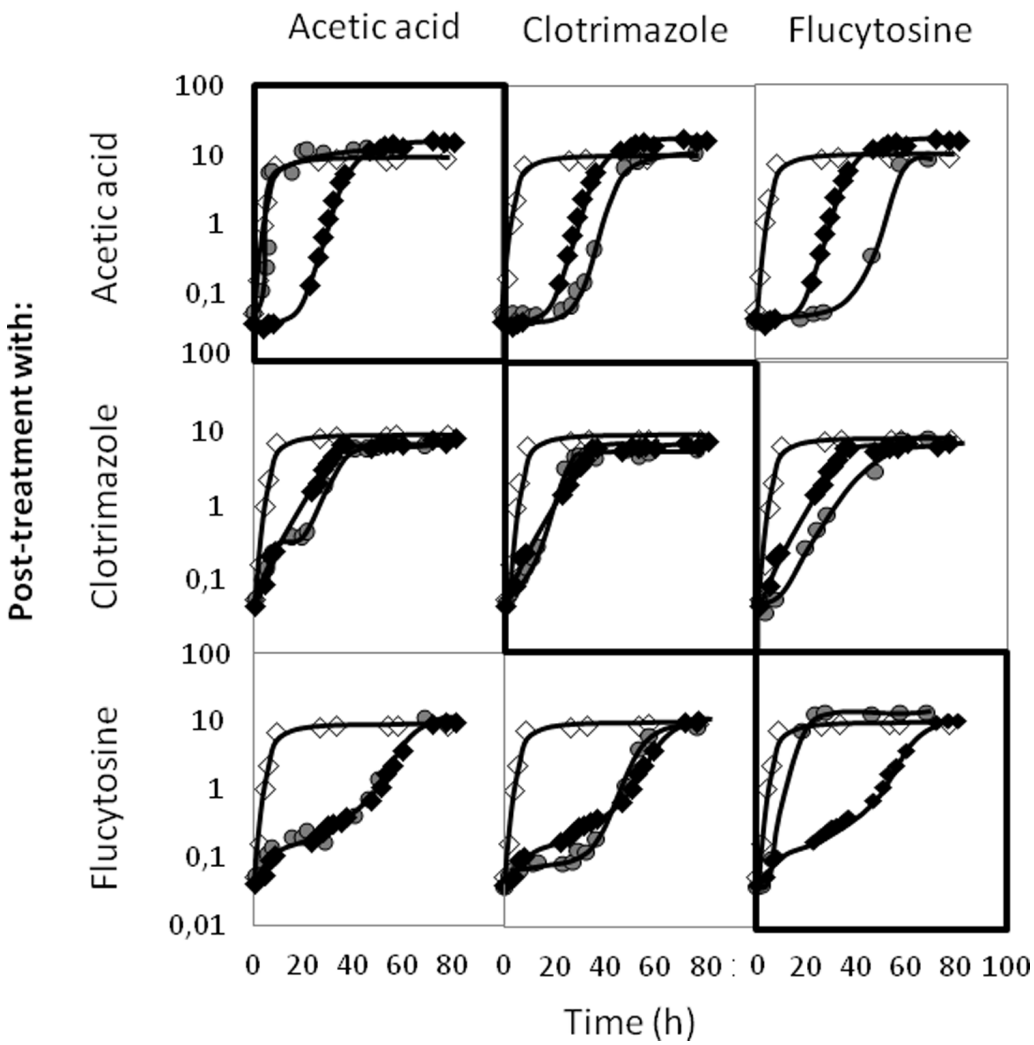

FIGURE 9 | Comparison of the growth curves of *C. glabrata* KUE100 strain, in liquid BM medium, in the absence (◊) or presence of acetic acid, clotrimazole, or flucytosine (♦,•), as indicated on the left. The inocula were prepared as described in Section "Materials and Methods," from exponentially growing cells that were growing in control conditions (♦), or that were pre-adapted (•) to the stress inducer indicated on the top. The displayed growth curves are representative of at least three independent experiments.

that in turn affect the plasma membrane partition, permeability or toxicity of drugs, and xenobiotics (Vargas et al., 2007; Cabrito et al., 2011; Teixeira et al., 2011, 2012). Such a possible scenario can also be envisaged to explain how CgAqr1 may confer acetic acid resistance, without affecting its intracellular accumulation.

Tolerance against weak acids is an important feature for *Candida* species to thrive in the acidic environment (pH ∼ 4) of the vaginal tract, where significant concentrations of acetic and lactic acids can be found (Davis, 2009). Furthermore, *C. glabrata* is able to survive inside the phagolysosome, where it has to deal with hydrolytic enzymes and an acidic environment. Results from this study point out to the existence of a synergistic action between both flucytosine and clotrimazole and acetic acid, a phenomenon observed previously between acetic acid and fluconazole in *C. albicans* (Moosa et al., 2004), reinforcing the importance of weak acid resistance mechanisms in the context of antifungal therapy. This effect, coupled with the dual role of CgAqr1 in acetic acid and antifungal drug resistance led us to hypothesize that pre-exposure of *C. glabrata* cells to acetic acid concentrations similar to those found in the vaginal tract might contribute to make them more tolerant to antifungal drugs. However, the predicted cross-resistance effect was not observed. Indeed, pre-exposure to acetic acid had no effect on the susceptibility of *C. glabrata* cells to flucytosine or clotrimazole, whereas pre-exposure to clotrimazole and flucytosine sensitizes these cells toward acetic acid stress. Interestingly,

CgAqr1 transcript levels in *C. glabrata* were seen to be irresponsive to chemical stress exposure, either that induced by acetic acid, flucytosine, or clotrimazole, suggesting that other genes or post-translation regulatory effects may underlie the antifungal drug-induced sensitization of *C. glabrata* cells. The fact that CgAqr1 confers resistance to both antifungal drugs and acetic acid strongly suggests that this transporter may play an important role in the persistence of *C. glabrata* infections in acidic loci.

Interestingly, the deletion of *CgAQR1* was found to play a major role in decreasing stress-induced lag-phase duration, while having only a moderate effect in *C. glabrata* maximal growth rate in the presence of flucytosine, acetic acid, or clotrimazole. A similar phenomenon was observed for most of the CgAqr1 homologs in *S. cerevisiae* (Sá-Correia et al., 2009). The current model to explain this phenotype proposes that drug efflux pumps are mostly required to deal with sudden exposure to chemical stress. Additional mechanisms leading to the impermeabilization of the cell envelope are reported to be activated upon chemical stress exposure, preventing drug re-entrance, and relieving the requirement for the energy expensive process of extruding drugs (Simões et al., 2003; Viegas et al., 2005). This model justifies the fact that drug efflux pumps appear to be required to a lower extent during exponential growth in the presence of stress, even when the stress agent is not degraded or neutralized. Indeed, no catabolism of clotrimazole or acetic acid, in the presence of glucose, is registered in yeast cells (Prasad et al., 2002; Guerreiro et al., 2012). Thus, when a new population is suddenly exposed to the vaginal environment or antifungal therapy or, more significantly, the two stresses at the same time, the expression of Aqr1 appears to be important to the success of colonization and persistence, but is not the single factor leading to chemical stress resistance.

This study, characterizing the *C. glabrata* Aqr1 multidrug transporter involved in flucytosine and azole drug resistance, highlights the importance of studying the remaining members of this family in *C. glabrata* in this context, with an expected impact in the treatment of the increasing number of drug resistant fungal infections. Significantly, CgAqr1 has close homologues in other pathogenic *Candida* species, e.g., orf19.9520 from *C. albicans*, CPAR2_501800 from *C. parapsilosis* or Cd36_51250 from *C. dubliniensis*, which may also play a role in acetic acid and antifungal drug resistance in these related pathogenic yeasts. So far, only the drug:H^+ antiporters Mdr1 and Flu1, from *C. albicans* (Goldway et al., 1995; Calabrese et al., 2000), CgQdr2 from *C. glabrata* (Costa et al., 2013) and Mdr1 from *C. dubliniensis* (Sullivan et al., 2004) have been linked to antifungal drug resistance, more specifically to azoles. In *C. glabrata*, there are 15 predicted DHA transporters, of which 10 belong to the DHA1 family, predicted to have 12 transmembrane spanners, and 5 to the DHA2 family, predicted to have 14 transmembrane segments (Gbelska et al., 2006). The expectation that these transporters, that remain so far mostly uncharacterized, may play a significant role in multidrug resistance in *C. glabrata* is reinforced by the findings of this study.

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Sorbic acid stress activates the *Candida glabrata* high osmolarity glycerol MAP kinase pathway

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Weak organic acids such as sorbic acid are important food preservatives and powerful fungistatic agents. These compounds accumulate in the cytosol and disturb the cellular pH and energy homeostasis. *Candida glabrata* is in many aspects similar to *Saccharomyces cerevisiae*. However, with regard to confrontation to sorbic acid, two of the principal response pathways behave differently in *C. glabrata*. In yeast, sorbic acid stress causes activation of many genes via the transcription factors Msn2 and Msn4. The *C. glabrata* homologs CgMsn2 and CgMsn4 are apparently not activated by sorbic acid. In contrast, in *C. glabrata* the high osmolarity glycerol (HOG) pathway is activated by sorbic acid. Here we show that the MAP kinase of the HOG pathway, CgHog1, becomes phosphorylated and has a function for weak acid stress resistance. Transcript profiling of weak acid treated *C. glabrata* cells suggests a broad and very similar response pattern of cells lacking CgHog1 compared to wild type which is over lapping with but distinct from *S. cerevisiae*. The *PDR12* gene was the highest induced gene in both species and it required CgHog1 for full expression. Our results support flexibility of the response cues for general stress signaling pathways, even between closely related yeasts, and functional extension of a specific response pathway.

Keywords: sorbic acid, HOG pathway, Candida glabrata, stress response, Fungal pathogen

INTRODUCTION

Candida species cause mucosal as well as disseminated infections in humans. Many women experience Candida vulvovaginitis, with recurring infections (Buitron Garcia-Figueroa et al., 2009). *Candida albicans* is the leading cause of such infections, with *Candida glabrata* accounting for about 20% (Schmalreck et al., 2012). Compared to *C. albicans*, *C. glabrata* is more similar to *Saccharomyces cerevisiae* (Dujon, 2010) and might be regarded as a Saccharomycete with adaptations to mammalian commensalism. Striking adaptations are the optimal growth temperature of 37°C, its short division time of about an hour at this temperature (Roetzer et al., 2011), and the ability to adhere to various surfaces due to a number of adhesins (Domergue et al., 2005). *C. glabrata* has a characteristic high resistance to certain common antifungal drugs such as azoles (Cross et al., 2000; Pfaller and Diekema, 2007). All *C. glabrata* isolates are haploid suggesting an absent or greatly reduced sexual cycle. The inability to differentiate into spores as a resistant cell type is perhaps outweighed by high persistence due to stress and drought resistance (Berila and Subik, 2010).

Adjustment of metabolism and cell cycle to changing environmental conditions is essential for microbial organisms. Evidently, a pathogen has to gather specific information about its host environment (Shapiro et al., 2011). Fungi communicate and respond to their environment with an array of signaling components such as MAP kinase pathways (Chen and Thorner, 2007), dedicated transcription factors such as the heat shock transcription factor Hsf1 or the copper responsive factor Ace2, and conserved signaling pathways such as the protein kinase A (PKA), target of rapamycin (TOR), DNA damage responsive pathways and many more. Fungal cells use this information to make decisions to pass cell division checkpoints and for channeling of resources toward defence or growth (Zakrzewska et al., 2011). Thus, environmental response signaling pathways are one putative Achilles' heel for intervention with fungal growth and establishment in environmental niches provided by the host. In fact, some combinations of stress types are efficiently preventing fungal growth (Kaloriti et al., 2012).

During antifungal therapy mutations arise in special genes such as drug efflux pumps and their regulators due to selective pressure (Ferrari et al., 2011a,b). However, the clue to the success of *C. glabrata* lies possibly in its ability to persist. The intrinsic stress resistance of *C. glabrata* likely has a multigenic basis and a dynamic component which can be established by external conditions and might even be propagated by epigenetic mechanisms. The transient aspect of resistance traits of clinical isolates has not been explored systematically.

The well-characterized high-osmolarity glycerol (HOG) pathway is essential for yeast survival under-high osmolarity conditions, since it triggers adaptation through intracellular

accumulation of glycerol as the adaptive osmolytes to reestablish the balance of water and ion concentration (De Nadal and Posas, 2010). Yeast Hog1 becomes activated by dual phosphorylation and translocates from the cytosol to the nucleus to change gene expression patterns. In addition, Hog1 has cytosolic targets such as the Fps1 aquaglyceroporin (Thorsen et al., 2006; Mollapour and Piper, 2007). Stress activated protein kinase networks are well conserved across kingdoms and orthologs of Hog1 are present in other fungi (Nikolaou et al., 2009). However, the detailed action of Hog1 in *C. glabrata* and its downstream targets have not been investigated in depth so far. Hog1 is related to the p38 MAP kinase of higher eukaryotes. There, this kinase has a broader role as initially described for *S. cerevisiae* Hog1 which is mainly responsive to osmolarity changes. However, other stress types such as oxidative stress as well as exposure to methylglyoxal (Aguilera et al., 2005) or acetic acid (Mollapour and Piper, 2006) activate this pathway. In *C. albicans*, Hog1 becomes activated by oxidative stress and Cadmium exposure (Enjalbert et al., 2006; Yin et al., 2009). In yeast and *C. glabrata*, the HOG signaling pathway is required for osmotic stress response and oxidative stress (Kaloriti et al., 2012). In contrast to yeast, however, CgPbs2 (the MAPKK) mutants display hypersensitivity to weak organic acids (Gregori et al., 2007b). These results would suggest that CgHOG pathway compared to ScHog1 has acquired or retained other functions.

Short chain carboxylic acids or weak organic acids are in widespread use as preservatives for food and feed (Plumed-Ferrer and Von Wright, 2011). A recent excellent review covers the effects of weak acids on yeasts (Piper, 2011). Weak acid response at the transcriptional level is mediated by the zinc cluster transcription factor War1 (weak acid response 1) in *S.cerevisiae, C. albicans*, and *C. glabrata* (Kren et al., 2003; Lebel et al., 2006; Mundy and Cormack, 2009). Remarkably, War1 is not conserved in intrinsically highly weak acid resistant food spoiling Zygomycetes (Mollapour et al., 2008). In *S. cerevisiae* this factor triggers expression of a small regulon consisting of an ABC (ATP binding cassette) transporter gene *PDR12* which is required for weak acid ion efflux as well as an ammonia transporter *FUN34* (Kren et al., 2003; Schüller et al., 2004; Gregori et al., 2007a). Strikingly, activation of *PDR12* transcription is sufficient for weak acid resistance (Schüller et al., 2004). Activation of War1 by weak acids is possibly rather direct, (e.g., interaction with the acid or a metabolite) since no regulatory factors upstream of War1 have been identified to date by genetic means. Attempts to assign a regulatory function to weak acid induced changes of the War1 phosphorylation status, which results in a robust migration difference, were unsuccessful (Frohner et al., 2010; Mollapour and Piper, 2012). It has been suggested that War1 becomes directly activated by weak acids triggering conformational changes (Gregori et al., 2008). In addition, adaptation to weak acids also requires Haa1, a further weak acid inducible transcription factor *S. cerevisiae* (Fernandes et al., 2005).

S. cerevisiae cells treated with weak organic acids accumulate the transcription factors Msn2 and Msn4 rapidly in the nucleus and activate a relative large regulon of generic stress responsive genes (Schüller et al., 2004). Remarkably, induction of the majority of sorbate-induced genes required Msn2/4, however, weak organic acid tolerance was unaffected by a lack of Msn2/4. Strikingly, CgMsn2 did not accumulate in *C. glabrata* cells exposed to weak acids (sorbate and propionate). However, CgMsn2 expressed in yeast cells showed similar nuclear accumulation as ScMsn2 (Roetzer et al., 2008). Thus, *C. glabrata* either lacks a pathway which is operative in *S. cerevisiae* or responds differently to weak acids. For example, different cellular acidification of weak acid exposed *S. cerevisiae* or *C. glabrata* cells could play a role (Ullah et al., 2013b). Alternatively, weak acids might affect growth and thus Msn2/4 activation indirectly (Zakrzewska et al., 2011). Weak acids induce expression of the *C glabrata* adhesin Epa6 via an Msn2/4 and War1 independent mechanism leading to enhance adherence. This is remarkable because use of weak acids is widespread as preservative (not as the active ingredient) in over-the-counter vaginal products (Mundy and Cormack, 2009).

We report the generation of a *C. glabrata* strain deleted for *HOG1*. We find that sorbic acid stress causes a robust high level phosphorylation of CgHog1 coinciding with an enrichment in the nucleus. Moreover, we show that CgHog1 is required for sorbic acid resistance of *C. glabrata*.

MATERIALS AND METHODS

For gene disruption of *HOG1* in the BG14 background, we used the *SAT1* nourseothricin resistance marker cassette amplified from plasmid pSFS2 (Reuss et al., 2004). Via three-way PCR a knockout cassette with long homologous flanking regions was created and integrated into the BG14 strain. Correct insertion of the cassette was verified by genomic PCR. Primer sequences: Hog1–1 5′GGC TAC TAA TGA AGA GTT CAT AAG, Hog1–2 5′cac ggc gcg cct agc agc ggC TAC TCC TGC TGA GTG AAC G, Hog1–3 5′gtc agc ggc cgc atc cct gcC AGA GGC AAA GTT TGA CTG G, Hog1–4 5′CAC TGC TTG ATT AGC ATA CTC. To determine susceptibilities to osmostress and sorbic acid exponentially growing cultures were adjusted to an optical density at 600 nm (OD_{600}) of 0.1 and diluted 1:10, 1:100, and 1:1,000. Equal volumes of serial dilutions were spotted onto YPD (pH 4.5, adjusted with HCl) plates containing various concentrations of NaCl and potassium sorbate. Plates were incubated at 30°C for 36–48 h.

HOG1 PHOSPHORYLATION ANALYSIS

The phosphorylated Hog1 isoforms were detected using two different anti-phospho-p38 MAPK antibodies as indicated in **Figure 3**. Phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling Technologies #9211) recognizes either Thr180 or Tyr182 phosphorylated species while antibody ab4822 (Abcam) recognizes double Y182 + T180 phosphorylated species, according to the manufacturers. Cells were grown to OD_{600} of 1 treated for 40 min with potassium sorbate, harvested and frozen. TCA extracts were prepared, and cell lysates equivalent to 0.5 OD_{600} unit were fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% SDS-PAGE) and transferred to nitrocellulose membranes. ScPgk1 antibodies were used to detect CgPgk1 as loading control as described (Gregori et al., 2007a), or a cross reaction served as loading control. The *CgHOG1* open reading frame was cloned into the unique BamHI site of vector pGRB2.2 using sites introduced by PCR immediately upstream of the start codon and replacing the stop codon (Mumberg et al., 1995; Frieman et al., 2002) and fused to yEGFP originating from

pKT128 (Sheff and Thorn, 2004) introduced with a unique EcoRI in front of the start codon and a SalI site after the stop codon.

MICROSCOPY

Cells expressing GFP tagged variants of CgHog1 were grown to mid-exponential phase. Appropriate cultures were visualized live with a CELL R system (Olympus, Japan) and detected with an Orca R2 camera (Hamamatsu Photonics K.K. Japan). Quantification of the fluorescence intensity was done with ImageJ. The background subtracted signal ratio of cytosol to nucleus was determined comparing the fluorescence signals obtained from both compartments during 10 min 10 mM of sorbic acid stress, and non-stress conditions.

EXPRESSION PROFILING

For microarrays, BG14 and BG14*hog1*Δ strains were grown for 4 generations in 50 ml cultures in YPD at 30°C to $OD_{600\,nm}$ of about 1 before potassium sorbate was added to a final concentration of 20 mM. Three biological replicates were generated. After 20 min cells were harvested and immediately frozen. RNAs were prepared as described earlier (Klopf et al., 2009)1 μg of total RNA was used for labeling reaction (Agilent Quick Amp Labeling Kit, two-color, Cat.Nr. 5190–0444). The standard Agilent Protocol for one-color labeling was used (G4140–90041). 325 ng of Cy3 labeled cRNA were hybridized to the custom *C. glabrata* GE 8 × 15 K arrays [Agilent eArray Design 017617; GPL10713 (Ferrari et al., 2011b)]. Samples were hybridized for 17 h at 65°C, 10 rpm. Agilent G2505C Microarray Scanner System was used to scan the arrays. The Agilent Feature Extraction program (Version FE 10.5.1.1) was used to analyze the array images. Values were normalized with quantile scale transformation at Babelomics (http://babelomics.bioinfo.cipf.es) using the Bioconductor affy package. Duplicate features were averaged. Features with gProcessedSignal values >8 (log2) (equals ∼10 fold signal over background) were retained and per condition average of 2 and 3 values further analyzed. GO terms enriched in selected sets were extracted via String (string-db.org) and GOTermfinder (yeastgenome.org). Analyzed with Cluster analysis, using cluster3 and visualized with TreeView (Saldanha, 2004) (http://jtreeview.sourceforge.net). TreeView files corresponding to the figures and the raw data are supplied (Supplematary Datafile1). Data have been submitted to GEO (accession No. GSE52382).

qRT-PCR measurement of *PDR12* transcript levels of cells treated with 20 mM sorbic acid for 20 min at neutral pH. Poly T anchored cDNA was synthesized by using Revert Aid Reverse Transcriptase (Thermo Scientific). *PDR12* cDNA was detected using RT-Primers within the coding sequence Pdr12-RT (for.) GGAAAGGAAGGATGATGCAGA and Pdr12-RT (rev.) CTG GCCATGGACTCCAATCTT. *ACT1* was used as a stress unresponsive internal control and amplified via Act1-RT (for.) ATC GTTTCCCCCTTTGCCAC and Act1-RT (rev.) TGCCCACCA CTCCTAACTCA.

RESULTS

The *C. glabrata HOG1* (CAGL0M11748g) gene was deleted in the BG14 background (Mundy and Cormack, 2009) by homologous recombination and replaced with the nourseothricin resistance gene. It was not possible to recover mutants with eliminated open reading frame due to high background of false positives caused by frequent random integration of the corresponding disruption cassette. We were successful with the deletion of the region between ORF base pair positions 402–960 eliminating the coding sequence for the active site of the kinase domain. The correct integration of the cassette was verified by genomic PCR (**Figure 1A**). The Cg*HOG1* deleted strain, BG14*hog1*Δ, was sensitive to high osmolarity conditions higher than 0.5 M NaCl (**Figure 1B**). The *C. glabrata* mutant lacking Hog1 could tolerate higher salt concentration than the corresponding *S. cerevisiae* mutant strain. In addition to the expected phenotype of high osmolarity sensitivity, BG14*hog1*Δ, was sensitive to sorbic acid stress (**Figure 2A**). We found reduced growth on pH4.5 medium containing 1 mM sorbic acid and severely reduced growth with 3 mM, as observed earlier (Gregori et al., 2007b). At neutral pH, concentrations above

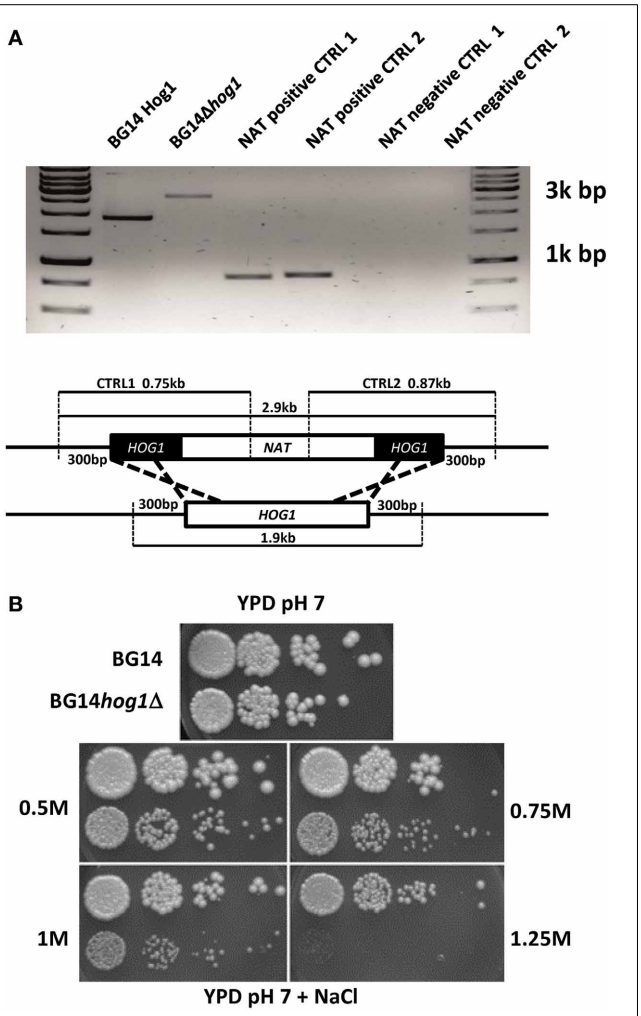

FIGURE 1 | (A) Confirmation of the integration of the marker cassette in to the Cg*HOG1* locus. The genomic locus and primer binding sites is indicated. **(B)** *C. glabrata* Hog1 is required for osmotic stress tolerance. BG14, BG14*hog1*Δ, and BG14 carrying pHog1-GFP were diluted and spotted on YPD containing the indicated amount of NaCl. Growth was recorded after about 36 h.

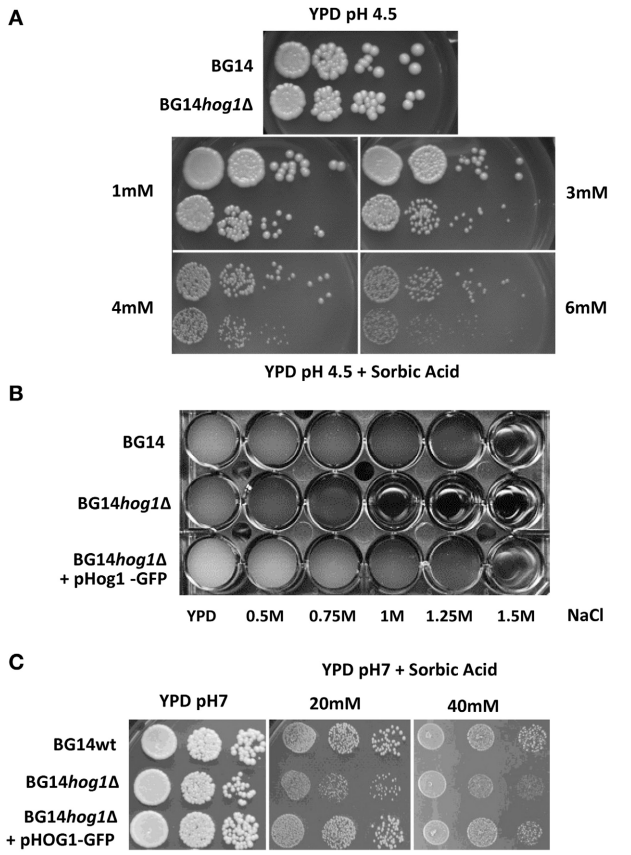

FIGURE 2 | (A) *C. glabrata* mutants deleted for *HOG1* display sensitivity to weak acids. Cultures of the *C. glabrata* strains BG14 and BG14*hog1*Δ, were diluted and spotted onto rich medium containing the indicated amounts of sorbic acid at pH 4.5. Growth was recorded after about 36 h for the control and 48 for weak acid stress. **(B)** CgHog1-GFP complements the osmotic stress sensitivity of the BG14*hog1*Δ strain. Cultures of BG14, BG14*hog1*Δ and BG14*hog1*Δ transformed with CgHog1-GFP were diluted to OD_{600} of 0.01 in YPD and exposed to a range of NaCl concentrations. Growth was recorded after 24 h. **(C)** CgHog1 is required for growth under sorbic acid stress. Cultures of wild type and BG14*hog1*Δ cells with and without CgHog1-GFP were diluted and spotted onto medium containing the indicated amounts of sorbic acid at neutral pH. CgHog1-GFP complements the growth defect of BG14*hog1*Δunder sorbic acid stress. Growth was recorded after 36 h of incubation.

20 mM sorbic acid were required to inhibit growth (**Figure 2C**). The overall sensitivity of the *HOG1* deleted strain was similar to the mutant lacking the MAPKK Pbs2 (Gregori et al., 2007b). Notably, *S. cerevisiae* strains lacking Hog1 are not sensitive to sorbic acid.

We next investigated if CgHog1 is activated due to weak acid stress exposure. Activation of Hog1 by phosphorylation of the activation loop residues by the MAPKK Pbs2 causes its rapid translocation into the nucleus. A fusion gene of GFP to the Cg*HOG1* open reading frame was created and expressed from a plasmid. The CgHog1-GFP fusion construct complemented the deleted allele (**Figures 2B,C**). The fluorescence signal of the CgHog1-GFP construct showed a substantial nuclear accumulation in unstressed cells. A similar partial nuclear accumulation of Hog1-GFP in unstressed cells has also been observed in our lab in another *C. glabrata* strain background (CBS138). Partial nuclear localization CgHog1-GFP in unstressed cells is possibly due to a certain level of basal activation of CgHog1 in BG14 cells (**Figures 3C,D**). Despite an apparent partial activation in unstressed cells, exposure to sorbic acid led to an enhanced staining of nuclei by Hog1-GFP within short time. The microscopy pictures shown in **Figure 3** were scanned after about 10 min. However, Hog1-GFP nuclear accumulation was visible immediately after addition of sorbic acid.

To support a direct role of the HOG pathway during weak acid stress we determined the CgHog1 phosphorylation status. We analyzed phosphorylation of the CgHog1 activation loop with antibodies recognizing the p38 mono and double phosphorylated TEY motif. Interestingly, and different from *S. cerevisiae*, we found signals indicating phosphorylation and thus active CgHog1 in unstressed cells (**Figures 3C,D**). CgHog1 phosphorylation level increases in cells treated with 5 and 10 mM sorbic acid for 40 min at pH4.5 (**Figure 3C**). Both the endogenous CgHog1 and the plasmid encoded CgHog1-GFP fusion protein showed similar changes of phosphorylation levels upon treatment with sorbic acid. The signal originating from CgHog1 is identified by testing a BG14*hog1*Δ strain without GFP plasmid (**Figure 3D**). Interestingly, the GFP fusion protein splits into two species of which the slower migrating form shows more dynamic changes of phosphorylation. The signals obtained suggest that two pools of CgHog1 might exist in *C. glabrata* which are perhaps not separated at the level of the wild type size protein. One is apparently constitutively phosphorylated and the other shows dynamic changes. We repeated the experiment with an antibody recognizing the double phosphorylated form to confirm that the observations are not due to a mono-phosphorylated TEY, and thus likely inactive CgHog1. In addition we exposed the cells at neutral pH to sorbic acid to avoid additional stress. We detected an increase of CgHog1 and of CgHog1-GFP phosphorylation to a comparable level in the osmotic and sorbic acid stressed case (**Figure 3D**).

The consequence of Hog1 activation in *C. albicans* and *S. cerevisiae* is, apart from changes of the activity of cytosolic targets (Thorsen et al., 2006; Mollapour and Piper, 2007), nuclear translocation and a dramatic change of gene expression mediated by a set of transcription factors. We were interested to which extend CgHog1 is influencing transcriptional patterns of sorbic acid stressed *C. glabrata* cells. We used single color arrays as described in materials and methods and report the results from a 20 min treatment with 20 mM Sorbate at neutral pH of BG14 and BG14*hog1*Δ. The overall response pattern of repressed and induced genes in was very similar between mutant and wild type as seen in a clustered presentation and a scatter plot (pearson correlation coefficient $r = 0.84$ of genes in **Figure 4A**). The highest induced genes in both wild type and mutant were Cg*PDR12*, Cg*BTN1* (possible role in mediating pH homeostasis), Cg*HSP42* (a small heat shock protein with chaperone activity), Cg*DCS2* [m(7)GpppX pyrophosphatase regulator protein]. Further differentially regulated genes are listed in (Supplemantary DataSheet1/Top_changed). The repressed genes were enriched in protein synthesis related functions. We selected 466 genes

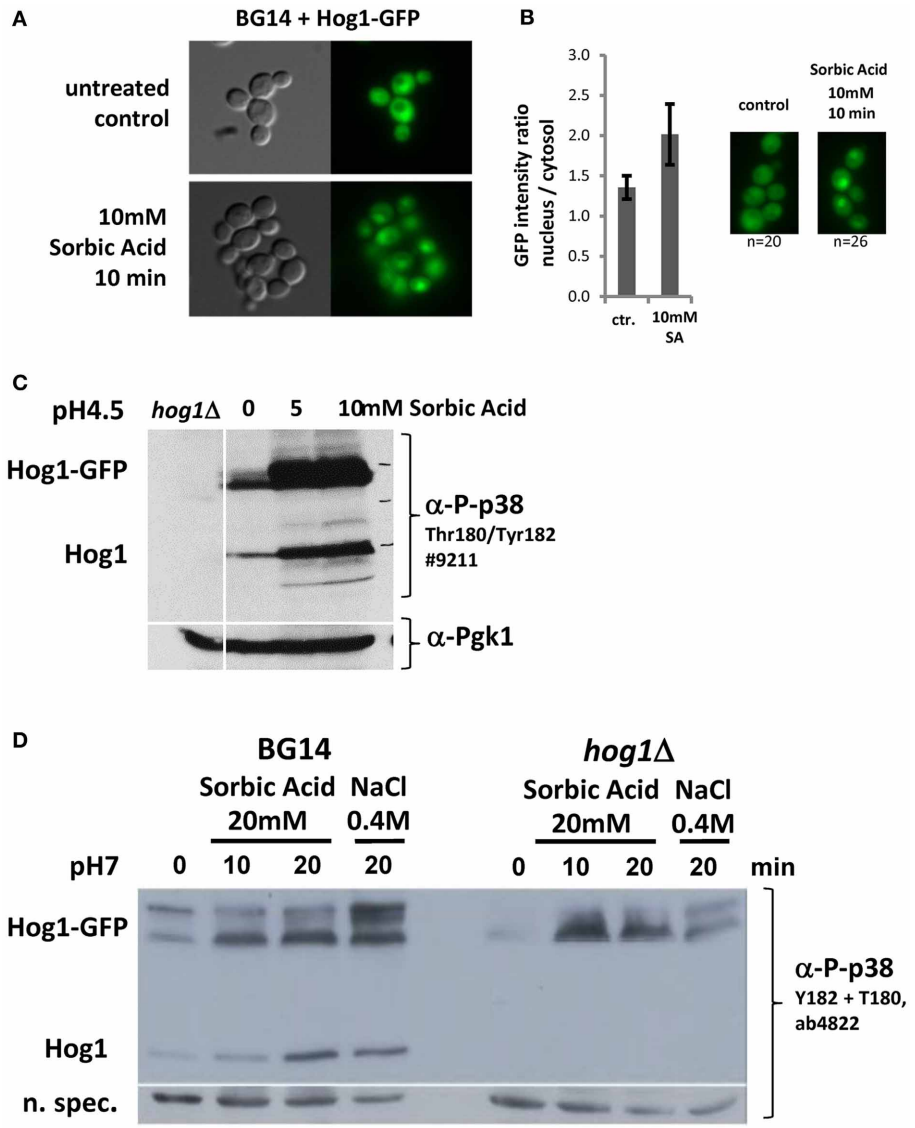

FIGURE 3 | (A) CgHog1 accumulation increases in the nucleus of sorbic acid stressed cells. Life Microscopy of BG14 expressing CgHog1-GFP without stress (upper panel) or under Sorbic acid exposure (10 min, 10 mM, lower panel). **(B)** Nuclear accumulation of Hog1 upon stress induction results in a higher fluorescence signal and increases the nucleus to cytosol signal ratio. Fluorescence intensity ratio, nucleus to cytosol, in unstressed ($n = 20$) and stressed ($n = 26$) cells. **(C)** CgHog1 and CgHog1-GFP phosphorylation increases in sorbate treated cells. Cultures of BG14, BG14*hog1*Δ, and BG14 carrying pHog1-GFP were grown in YPD (pH 4.5) to the early-exponential-growth phase before 5 or 10 mM Sorbic acid was added. Samples were harvested after 20 min. Detection was carried out using polyclonal anti-phospho-p38 MAPK or anti-Pgk1 antibodies. **(D)** Dual phosphorylation of Hog1 upon sorbic acid and osmotic stress. Cultures of BG14, BG14*hog1*Δ, and BG14 carrying pHog1-GFP were grown in YPD (pH7) to the early-exponential-growth phase. Sorbic acid was added to a final concentration of 20 mM, samples were taken after 10 and 20 min. Osmotic stress (0.4 M NaCl) for 10min was used to compare to a standard induced Hog1 phosphorylation pattern.

with induced expression (>2.25 fold) in wild type or mutant (**Figures 4B,C**). These can be divided into two clusters. Cluster one is enriched in genes with functions in proteolysis, autophagy and mainly cytosolic components. GO terms are reported in supplementary material (Supplemantary DataSheet1). For a small number of genes of this cluster, the fold increase of was reduced by a factor of more than 2 in absence of CgHog1 (Supp DataSheet1/Top_changed; *CAGL0L05016g STB6*, *CAGL0K07205g* (unknown), *CAGL0C02321g PHM8*, *CAGL0F01111g OPI10*, *CAGL0B00858g STE50*, *CAGL0K07590g*, *MYO3*). These genes have striking relations to starvation and stress resistance. Phm8 is a Lysophosphatidic acid (LPA) phosphatase active in response to phosphate starvation. Ste50 is involved in mating, filamentous growth and osmotolerance. If these or other transcripts changed in the mutant contribute to the sorbic acid sensitivity phenotype remains to be shown. Expression levels of transcription factors with a function for to stress response are rarely regulated by stress due to their required latent presence. Interestingly, *CgMSN4* is

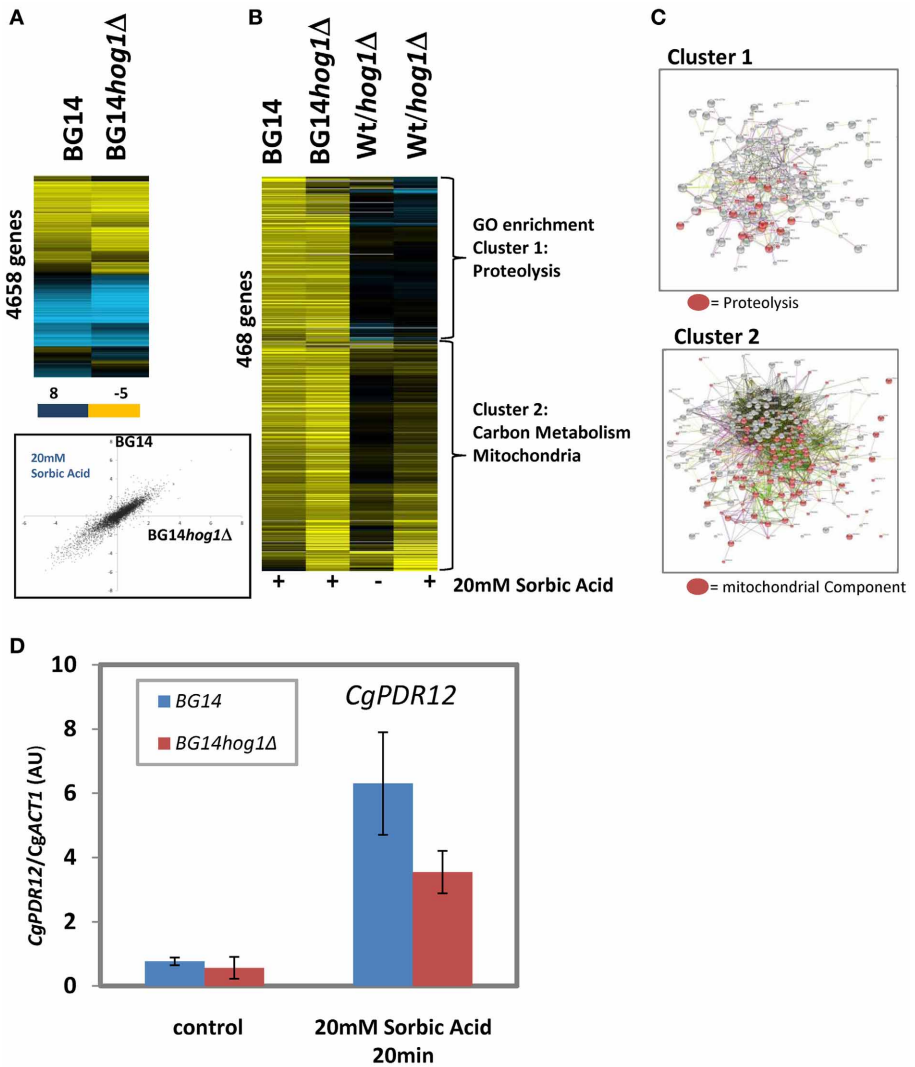

FIGURE 4 | Differentially expressed genes in BG14 and BG14*hog1*Δ cells, treated with 20 mM Sorbic acid for 20 min. (A) Overall comparison of gene expression changes in wild type and mutant. Scatter Plot of sorbate treated wild type vs. sorbate treated *hog1*Δ. **(B)** Clustering of selected genes with higher than 2-fold change of transcript level isolates two prominent clusters enriched in the indicated GO terms. **(C)** String connection maps of cluster one and two. **(D)** *CgPDR12* mRNA quantification after sorbic acid stress (20 mM, pH7, 20 min).

induced 5-fold (and higher in the mutant), whereas Cg*COM2* has a robust 2.6-fold increase of expression. Yer130C (designated *COM2* for Cousin Of Msn2) is a Zn-finger protein similar to ScMsn2/4 and *C. albicans* Mnl1 and interestingly its expression is induced by acetic acid stress (Mira et al., 2011). Therefore, Yer130C/Com2 might be connected to weak acid stress in both organisms.

Cluster two comprises the remaining genes of this set and is strikingly enriched in mitochondrial functions and carbon metabolism (Supplementary GO data in DataSheet1). Furthermore, cluster two is enriched in genes with lower expression levels in unstressed mutant cells. This result is indicated by String (http://string-db.org) network analysis. Comparison to *S. cerevisiae* sorbic acid stress data (Schüller et al., 2004; SuppTable1: SCvsCG_Sorbat) results in a mixed picture of a surprisingly weak correlation coefficient ($r = 0.25$), however, some characteristic similarities such as *PDR12* as the most highly induced gene exist. Inspection of the pattern of genes induced in both organisms raises a suspicion of less pronounced increase of *S. cerevisiae* Msn2/4 orthologous target genes in *C. glabrata*. However, part of the difference is of technical nature.

Failure to express the *CgPDR12* gene could contribute to the sensitivity of the CgHog1 deletion mutant. The *S. cerevisiae PDR12* gene confers most of weak acid resistance (Schüller et al., 2004). In our array data the *PDR12* gene changes more than 160-fold in the wild type and 130-fold in the mutant. We confirmed this by RT-qPCR in two independent biological replicas and found a fold change of >10-fold (due to higher unstressed background signal) and a similarly reduced level in the BG14*hog1*Δ strain (**Figure 4D**). Whether this mRNA level difference may

account for the sorbic acid sensitivity phenotype remains to be shown.

DISCUSSION

We report an initial characterization of the *C. glabrata* Hog1 MAP kinase and show activation of the HOG pathway by weak acids in this yeast. Weak acids like sorbic or benzoic acid traverse the plasma membrane in the protonated form and dissociate in the cytosol (Piper, 2011). The consequences of this influx are manifold. Yeast cells respond to weak acid stress with dedicated defense mechanisms like the War1-Pdr12 system but also to the indirect damage of weak acid accumulation. Previously, the *C. glabrata* HOG pathway has been shown to be involved in weak acid and acetate resistance (Gregori et al., 2007a). Acetate activates the pathway in both yeasts at concentrations above 50 mM. We show here that weak acid treatment causes a rapid increase of dually phosphorylated CgHog1 and thus signaling through this pathway. The sorbic acid stress sensitivity of the CgPbs2 mutant further indicates activation of the *C. glabrata* MAP kinase cascade but the sensing mechanism upstream is yet unknown (Gregori et al., 2007a). As an additional striking difference to *S. cerevisiae*, we observe a relatively high phosphorylation level of the CgHog1 kinase in unstressed cells. In contrast to ScHog1, and possibly connected to its basal phosphorylation, we find a partial accumulation of CgHog1 in the nucleus in unstressed cells. This could have consequences on the level of gene expression since our microarray experiment shows differences between the transcript patterns of unstressed deletion mutant and wild type. Basal expression of many genes linked to mitochondrial function and carbon catabolism is reduced in the mutant. The relevance of this observation to weak acid resistance or other phenotypes e.g. *C. glabrata* virulence remains to be investigated. In fact, the *in vivo* function of this characteristic might not at all be connected to the host environment. A constitutive partially active HOG pathway, which is not well tolerated by *S. cerevisiae*, could prepare *C. glabrata* cells for water deprivation and starvation *in vitro* (outside the host) and thus enhance persistence. A role for *S. cerevisiae* Hog1 for the re-entry into the cell cycle after starvation induced resting has been reported (Escote et al., 2011). Strikingly, Hog1 rapidly enters the nucleus at the time of resumption of growth in absence of osmotic stress. Since p38 shows similar response, this might represent a conserved SAPK-dependent response for cells leaving a resting state (Escote et al., 2011). ScHog1 activates some of its many target genes directly by interacting with Msn2 and other transcription factors. In this way ScHog1 becomes tethered to the respective target promoters and contributes substantially to gene regulation. CgMsn2 and CgMsn4 do not translocate into the nucleus in weak acid stressed *C. glabrata* cells, suggesting that they are presumably not involved (Roetzer et al., 2008). The cause for this difference to *S.cerevisiae* Msn2 is unclear, however, is not related to difference in intracellular acidification of sorbate exposed cells (Ullah et al., 2013b). Nevertheless, in *C. glabrata* a similar large number of genes change expression upon Sorbate treatment suggesting other factors substitute for their function. The sensitivity of the CgHog1 deletion mutant could originate from failure to express the *CgPDR12* gene. We think that this is not likely the case. Our gene expression dataset shows a large change of Cg*PDR12* transcript level. Cells lacking Hog1 had 2/3rd of wild type *CgPDR12* mRNA level and thus the HOG pathway does not contribute substantial to transcriptional regulation of the weak acid anion pump. Whether this mRNA level difference does cause part of the sensitivity phenotype remains to be shown. CgHog1 could also be involved in tuning membrane transport components and thus influence weak acid induced ATP expense, which has been reported recently to be regulated to prevent futile cycling (Ullah et al., 2013a).

Taken together, we show that weak acids trigger the *C. glabrata* HOG response pathway leading to MAP kinase activation and nuclear accumulation. Whether this interesting change of response pattern of the HOG pathway has relevance for virulence or persistence of *C. glabrata* requires further investigation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www. frontiersin.org/Microbial_Physiology_and_Metabolism/10.3389/fmicb.2013.00350/abstract

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Molecular mechanisms of *Saccharomyces cerevisiae* stress adaptation and programmed cell death in response to acetic acid

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Beyond its classical biotechnological applications such as food and beverage production or as a cell factory, the yeast *Saccharomyces cerevisiae* is a valuable model organism to study fundamental mechanisms of cell response to stressful environmental changes. Acetic acid is a physiological product of yeast fermentation and it is a well-known food preservative due to its antimicrobial action. Acetic acid has recently been shown to cause yeast cell death and aging. Here we shall focus on the molecular mechanisms of *S. cerevisiae* stress adaptation and programmed cell death in response to acetic acid. We shall elaborate on the intracellular signaling pathways involved in the cross-talk of pro-survival and pro-death pathways underlying the importance of understanding fundamental aspects of yeast cell homeostasis to improve the performance of a given yeast strain in biotechnological applications.

Keywords: yeast, acetic acid, cell adaptation, programmed cell death, mitochondrial retrograde pathway

INTRODUCTION

Acetic acid is a stress and death inducing agent produced *en route* to alcoholic fermentation carried out by *Saccharomyces cerevisiae*. Acetic acid can have negative effects in industrial fermentation processes such as wine production, negatively affecting wine quality (Garay-Arroyo et al., 2004; Vilela-Moura et al., 2010), or lignocellulosic fermentations for bioethanol production (Klinke et al., 2004; Liu and Blaschek, 2010; Mira et al., 2010b) underpinning its biotechnological relevance. For example, acetic acid concentration in grape must and wine may vary from 4 to even 80 mM, depending on its microbial origins (Antonelli et al., 1999; Vilela-Moura et al., 2010). Acetic acid is also a food preservative and the resistance of some yeast species to this compound can be associated with food spoilage causing major economic losses in the food industries (Stratford, 2006; Fleet, 2007). Thus the elucidation of the stress resistance and cell death mechanisms induced by acetic acid in yeast can impact the design of strategies for improving fermentations or decrease the food spoilage by acetic acid resistant-yeast species.

On the other hand, a fundamental aspect of acetic acid stress response is related to the capacity of the model organism *S. cerevisiae* to cope with newly encountered environmental conditions. Yeast may adapt and survive with alternatives in their genome expression and metabolism and is one of the most thoroughly studied unicellular eukaryotes at the cellular, molecular, and genetic level due to its well-known experimental tractability (Gasch and Werner-Washburne, 2002). Evidence has also been gathered showing that *S. cerevisiae* is able to undergo a programmed cell death (PCD) process triggered by different internal and external stimuli including acetic acid (Madeo et al., 1997, 1999; Ludovico et al., 2001, 2003). Such findings provide new tools and a model for cell death research at the molecular level (Carmona-Gutierrez et al., 2010). It is of note that mortality induced by acetic acid which accumulates in the culture medium has been proposed to participate in the mechanism of chronological aging in yeast; accordingly, buffering the medium to pH 6–7 significantly extends chronological life span (Burtner et al., 2009; Weinberger et al., 2010).

In this review we elaborate on current knowledge on the mechanisms of toxicity and tolerance to acetic acid stress obtained in the model eukaryote *S. cerevisiae*.

ACETIC ACID STRESS AND YEAST ADAPTATION

Like other weak acids, acetic acid displays increased antimicrobial action at low pH ($<pK_a = 4.76$) in the undissociated state (Lambert and Stratford, 1999). At pH 4.5 the uncharged molecules enter cells primarily by facilitated diffusion through the Fps1p aquaglyceroporin channel (Mollapour and Piper, 2007), encounter a more neutral pH in the cytoplasm and dissociate into acetate and protons (**Figure 1**). The protons lead to cytoplasmic acidification thereby inhibiting important metabolic processes (Arneborg et al., 2000). Weak acids induce activation of the proton-translocating ATPase Pma1p in yeast plasma membrane, which pumps out the protons generated by weak acid dissociation in the cytosol in an ATP-dependent manner. This ensures maintenance of the electrochemical potential across plasma membrane regulating ion and pH balance and providing energy for nutrient uptake (Carmelo et al., 1997; Martinez-Munoz and Kane, 2008; Ullah et al., 2012).

However, the differences in weak acid toxicity appear to mirror major differences existing in the transport and metabolism of the weak acid in yeast cells. Differently from sorbic and benzoic acid, which cannot be metabolized by *S. cerevisiae* and act

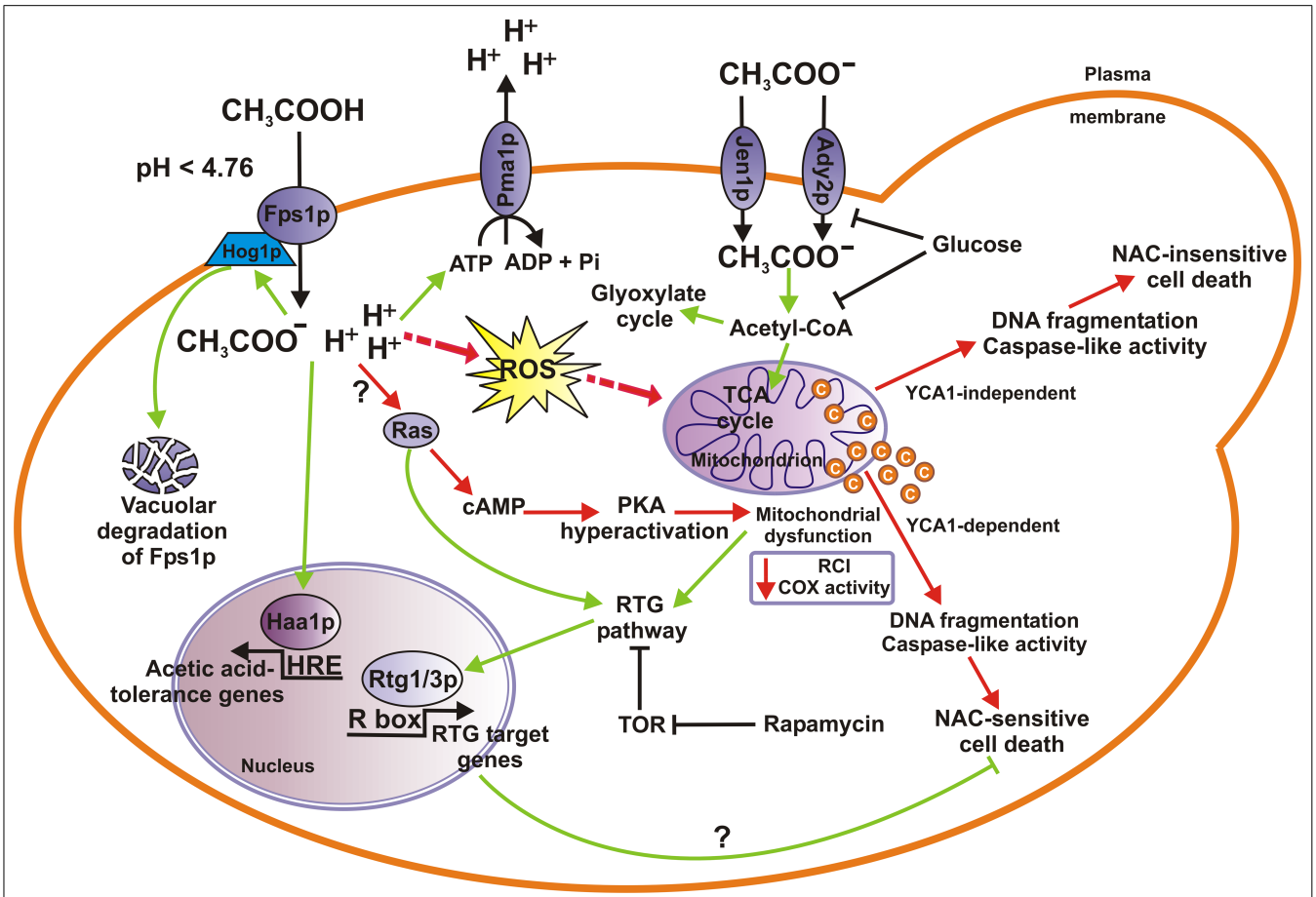

FIGURE 1 | Mechanisms of acetic acid stress response in *S. cerevisiae* cells. When yeast cells utilize acetic acid as the sole carbon source acetate anion enters cells through either Jen1p or Ady2p monocarboxylate transporter where it is converted into acetyl-CoA which enters the TCA or the glyoxylate cycle. Both acetate transport and metabolism are inhibited by glucose. At low pH ($pK_a = 4.76$), in the presence of glucose, acetic acid enters cells in its undissociated form by facilitated diffusion through Fps1p aquaglyceroporin channel, where more neutral cytosolic pH causes its dissociation into acid anions and protons. Concomitant cytoplasmic acidification by protons induces the activation of the Pma1p, a plasma membrane ATPase that pumps protons out of the cell. Acetic acid challenge may activate Hog1p, a MAP-kinase involved in phosphorylation and subsequent ubiquitination, endocytosis, and final vacuolar degradation of Fps1p, and transcription factor Haa1p enabling cells to adapt to varied levels of acetic acid. On the other hand, lethal concentrations of acetic acid induce ROS accumulation, cyt *c* release and mitochondrial dysfunction, caspase-like activity increase leading eventually to cell death (AA-PCD), with chromatin condensation and nuclear DNA fragmentation occurring as PCD hallmarks. AA-PCD can occur in *YCA1*-dependent or *YCA1*-independent manner. RTG signaling pathway is proposed to be activated in certain growth conditions causing AA-PCD resistance and cell adaptation to acetic acid stress (see text for details). RTG pathway is linked to TOR and Ras signaling pathways, where the former has an inhibitory effect on Rtg1/3-dependent gene expression, and the latter enhances retrograde response. Hyperactivation of Ras–cAMP–PKA pathway can lead to mitochondrial dysfunction, ROS production and apoptosis. Cell adaptation and acetate metabolic pathways (green arrows) and AA-PCD pathways (red arrows) are shown. COX, cyt *c* oxidase; RCI, respiratory control index; ROS, reactive oxygen species; TCA, tricarboxylic acid.

as membrane-damaging substances causing severe oxidative stress under aerobic conditions (Stratford and Anslow, 1998; Piper, 1999; Piper et al., 2001), acetic acid can be used as the sole carbon and energy source by *S. cerevisiae* and is not toxic under such conditions. Thus, *S. cerevisiae* cells are normally able to grow on acetic acid medium. Under this condition the weak acid is found in a dissociated form and acetate is transported across the plasma membrane either through an electroneutral proton symport transporter, encoded by *ADY2* (Casal et al., 1996; Paiva et al., 2004) or the monocarboxylate transporter encoded by *JEN1* (Casal et al., 1999). Acetate taken up by cells is converted to acetyl-CoA by one of either peroxisomal or cytosolic acetyl-CoA synthetases. Acetyl-CoA is then consumed in the glyoxylate shunt or oxidized in mitochondria through the tricarboxylic acid cycle (Vilela-Moura et al., 2008; Lee et al., 2011, and references Therein). However, typical *S. cerevisiae* cells grown on glucose cannot metabolize acetic acid due to the activation of glucose repression pathways (Rolland et al., 2002). Thus, yeast is sensitive to acetic acid stress in the presence of glucose. Acetate transport, as its metabolism, is also under glucose repression in *S. cerevisiae* but not in *Zygosaccharomyces bailii* that can metabolize acetic acid in the presence of glucose and is known for its high resistance to weak acids in glucose-containing media (Sousa et al., 1998; Rodrigues et al., 2012).

In glucose-containing media at pH 4.5 yeast cells can activate an adaptive response to weak acids, and resume to grow after a lag phase. Mechanisms of yeast adaptation to most common

monocarboxylate preservatives mainly involve plasma membrane transporters and proton-translocating ATPase. Plasma membrane transporter Pdr12p, a member of ATP-binding cassette (ABC)-transporter family was strongly induced by sorbic, benzoic acid, and certain other moderately lipophilic carboxylate compounds, and to a lesser extent by acetic acid. The accumulation of Pdr12p in the plasma membrane, dependent on War1p transcription factor (see below), increases weak acid resistance mediating cellular extrusion of weak acid anion (Hatzixanthis et al., 2003; Piper, 2011).

Transcription factor Haa1p is required for a rapid yeast adaptation to acetic and propionic acids (Fernandes et al., 2005; **Figure 1**). In particular, Haa1p, directly or indirectly, specifically regulates approximately 80% of the acetic acid-induced gene expression (Mira et al., 2010a,b,c, 2011). Among the Haa1p regulon, elimination of *HRK1* and, to a lower extent, of *SAP30* gene, led to the strongest susceptibility phenotypes to acetic acid, the first gene encoding a protein kinase possibly involved in the reduction of intracellular acetate concentration and the latter encoding a component of the Rpdl3L histone deacetylase complex involved in the epigenetic regulation of yeast transcriptional response to acetic acid stress (Mira et al., 2010a). Other transcription factors which are known to orchestrate weak acid stress response in yeast including Msn2p/Msn4p and Rim101p, regulate only a few number of acetic acid-tolerance gene transcription (Schuller et al., 2004; Mira et al., 2010c; Piper, 2011).

Unlike the sorbic acid stress, in which a gain of function of Pdr12p transporter is involved in the acid resistance through *PDR12* up-regulation, adaptation to acetic acid involves a loss of function (Mollapour et al., 2008 and references therein) of Fps1p aquaglyceroporin (**Figure 1**). Acetic acid challenge at low pH causes activation of two mitogen-activated protein (MAP) kinases, Hog1p, involved in the high-osmolarity glycerol (HOG) signaling pathway (Hohmann, 2009) and Slt2p involved in cell wall integrity pathway (Fuchs and Mylonakis, 2009). Hog1p-dependent phosphorylation of Fps1p results in its ubiquitination, endocytosis, and final degradation in the vacuole (Mollapour and Piper, 2007; Mollapour et al., 2009). Therefore, in a weak-acid specific manner, the Hog1p-directed destabilization of Fps1p eliminates the *route* for acetic acid entry into the cell, generating a resistance to varied levels of acetic acid (Piper, 2011; Zhang et al., 2011).

Such acetic acid stress response is different from hyperosmotic stress adaptation. At pH 6.8 on glucose medium cultures, very high concentrations of acetate anion (500 mM) inhibit yeast cell growth inducing a typical HOG response to sodium acetate salt stress with up-regulation of the expression of *GPD1*, encoding glycerol-3-phosphate dehydrogenase, and increased intracellular glycerol level to counteract hyperosmotic stress (Mollapour and Piper, 2006; Hohmann, 2009). At pH 4.5 a much lower acetic acid level (100 mM) is needed to cause comparable growth inhibition, with *GPD1* transcript displaying only a slight, transient induction and declining of intracellular glycerol (Mollapour and Piper, 2006). Yet, the transcription factors Gis1p and Rph1p, regulating glycerol and acetate metabolism, have been shown to function downstream of *TOR*, *RAS*/cAMP, and *AKT*/*SCH9* pathways in extending the lifespan of nutrient restricted yeast cells (Orzechowski Westholm et al., 2012).

ACETIC ACID-INDUCED PROGRAMMED CELL DEATH

Depending on their concentrations as well as on their lipophilic moiety, weak acids may cause delay of microbial cell growth, cytostasis, or cell death (Stratford and Anslow, 1996, 1998; Piper et al., 2001). Less lipophilic acetic acid under certain conditions, compromises cell viability leading cells to death (Pinto et al., 1989; Ludovico et al., 2001).

The yeast *S. cerevisiae* undergoes a PCD process in response to lethal concentrations of acetic acid. Recent achievements in the characterization of cell components and mechanisms involved in yeast acetic acid-induced PCD (AA-PCD) are reported below (**Figure 1**).

Since the discovery of a yeast mutant exhibiting apoptosis hallmarks (Madeo et al., 1997), *S. cerevisiae* has been established as an ideal model system to study PCD pathways due to the high level of phylogenetic conservation of biochemical pathways and regulators between yeast and mammals (Carmona-Gutierrez et al., 2010). Yeast PCD shares most of the morphological and biochemical hallmarks of mammalian apoptosis, such as phosphatidylserine externalization to the outer layer of the cytoplasmic membrane, DNA fragmentation, chromatin condensation, reactive oxygen species (ROS) production as well as a pivotal role of mitochondria (Eisenberg et al., 2007; Pereira et al., 2008; Guaragnella et al., 2012).

Exponentially growing *S. cerevisiae* cells undergo PCD when exposed to 80 mM acetic acid (Ludovico et al., 2001; Giannattasio et al., 2005a). Progressive loss of viable cells is complete after 200 min from AA-PCD induction. Consistently, AA-PCD cells showed early chromatin condensation with intact plasma membrane together with ribosomal RNA degradation; nuclear DNA fragmentation ensues, with the maximum percentage at 150 min (Guaragnella et al., 2006; Ribeiro et al., 2006; Giannattasio et al., 2008; Mroczek and Kufel, 2008). The earliest event (15 min) following acetic acid challenge is ROS production, with a different role for hydrogen eroxide and superoxide anion (Guaragnella et al., 2007). Hydrogen peroxide appears to be a second messenger in AA-PCD cascade of events, as also shown by AA-PCD inhibition by ROS scavenger N-acetyl cysteine (NAC; Guaragnella et al., 2010b). ROS level *en route* to AA-PCD is modulated by the antioxidant enzymes catalase and superoxide dismutase (SOD), whose over-expression prevents and exacerbates AA-PCD, respectively (Guaragnella et al., 2008).

Mitochondria are strongly implicated in AA-PCD. Following AA-PCD induction the release of cytochrome *c* (cyt *c*) starts at 60 min and reaches a maximum at 150 min. Cyt *c* is released from intact coupled mitochondria and once in the cytosol can function both as an electron donor and a ROS scavenger. Later in AA-PCD released cyt *c* is degraded, possibly by yet unidentified proteases and mitochondria become gradually uncoupled as judged by a decrease of the respiratory control index (RCI), a collapse of the mitochondrial membrane potential, a reduction in cyt c oxidase (COX) activity and in cytochromes a–a_3 levels (Ludovico et al., 2002; Giannattasio et al., 2008). Studies on ADP/ATP carrier, *YCA1* and cyt *c* knock-out cells have revealed that AA-PCD can also occur without cyt *c* release, but with a lower death rate compared to wild type cells (Pereira et al., 2007; Guaragnella et al., 2010a). Studies on mutant cells expressing a stable but catalytically inactive form of

the protein suggested that mitochondrial cyt *c* in its reduced state modulates AA-PCD and this occurs independently on its function as an electron carrier (Guaragnella et al., 2011b).

Yeast cells have a single gene, *YCA1*, encoding a type I metacaspase that was first implicated in the execution of oxidative stress-induced PCD (Madeo et al., 2002; Wilkinson and Ramsdale, 2011). AA-PCD can occur via two alternative pathways, one dependent and the other independent of *YCA1*. The two pathways differ one from another since the latter occurs without cyt *c* release, which requires *YCA1*, and is not sensitive to the antioxidant NAC (**Figure 1**). *YCA1* participates in the AA-PCD in a manner unrelated to caspase-like activity increase which is the latest event of AA-PCD occurring at 200 min from death induction (Guaragnella et al., 2006, 2010a,b, 2011a). *YCA1* also exerts a non-death role contributing to clearance of insoluble protein aggregates over the natural yeast lifespan promoting its longevity and fitness (Lee et al., 2008, 2010).

Interestingly enough, Gup1p, an O-acyltransferase required for several cellular processes including lipid metabolism and membrane remodeling, is required for AA-PCD to occur with Δ*gup1* cells dying by necrosis in response to acetic acid or in chronological aging (Tulha et al., 2012).

THE MITOCHONDRIAL RETROGRADE PATHWAY IN YEAST CYTOPROTECTION

Acetic acid stress sensitivity of yeast cells strongly depends on the extracellular environment. Indeed, when AA-PCD is induced in yeast cells growing on glucose as carbon source at pH 3.0, it has been shown that 30 min pre-conditioning in pH 3.0 medium set by HCl prior to acetic acid administration protects *S. cerevisiae* cells from AA-PCD (Giannattasio et al., 2005a). Since acetic acid is absent in the pre-conditioning medium, the hypothesis that the Hog1p-dependent degradation of Fps1p, described in Section "Acetic Acid Stress and Yeast Adaptation," could be involved in acid pre-conditioning (Mollapour and Piper, 2007; Mollapour et al., 2008) should be ruled out.

Instead, differently from AA-PCD cells, in acid stress-adapted cells acetic acid treatment does not cause any increase in intracellular ROS production (Giannattasio et al., 2005a; Guaragnella et al., 2007). Since mitochondria are the main source of ROS and a decline of mitochondrial function is observed *en route* to AA-PCD (Giannattasio et al., 2008), activation of mitochondrial stress response might be hypothesized under acid stress adaptation. **Figure 1** shows certain signaling pathways involved in cell response to mitochondrial dysfunction that may have a role in the cross-talk between cell death and adaptation mechanisms activated by acetic acid stress in yeast. The best characterized mechanism of cell response to mitochondrial dysfunction is the retrograde (RTG) pathway. Components and molecular details of RTG pathway have been better characterized in yeast (Butow and Avadhani, 2004; Liu and Butow, 2006). RTG-target gene expression is largely increased in cells with compromised mitochondrial function, such as cells lacking mitochondrial DNA (ρ^0; Liao et al., 1991). Rtg1p and Rtg3p are transcription factors that interact as a heterodimer to bind target sites called R boxes (GTCAC) located in the promoter region of the RTG target genes (Jia et al., 1997). Activation of Rtg3p correlates with its partial de-phosphorylation and its translocation with Rtg1p from the cytoplasm to the nucleus (Sekito et al., 2000). Rtg2p acts upstream of the Rtg1/Rtg3p complex, being both a proximal sensor of the mitochondrial dysfunction and a transducer of mitochondrial signals controlling Rtg1/3p nuclear localization through the reversible binding with Mks1p, a negative regulator of the RTG pathway (Uren et al., 2000; Liu et al., 2003, 2005). Other positive and negative regulators of the RTG pathway include Bmh1p, Bmh2p, Grr1p, and Lst8p (Liu et al., 2001, 2003, 2005; Giannattasio et al., 2005b). Hog1p has been shown to control Rtg1/3p nuclear localization and to phosphorylate Rtg3p upon osmostress (Ruiz-Roig et al., 2012). Activation of the RTG pathway leads to up-regulation of a subset of nuclear genes whose products function in anaplerotic pathways, fatty acid oxidation, and glyoxylate cycle (Butow and Avadhani, 2004; Liu and Butow, 2006).

It is of note that the RTG pathway is linked to other signaling pathways, such as target of rapamycin (TOR) pathway, which regulates cell growth in response to nutrient availability, and it has been reported to inhibit Rtg1/3-dependent gene expression (Komeili et al., 2000). However, it is clear that these two pathways do not overlap but act in parallel to converge on Rtg1/3p (Giannattasio et al., 2005b). The RTG response is also related to the Ras–cAMP signaling pathway (Jazwinsky, 2003). The inappropriate activation of PKA can lead to the production of dysfunctional, ROS generating mitochondria, and apoptosis (Colombo et al., 1998; Lastauskiene and Citavicius, 2008; Leadsham and Gourlay, 2010; **Figure 1**). In this context, it is of note that both TOR and Ras–cAMP–PKA signaling pathways are causally involved in yeast AA-PCD (Phillips et al., 2006; Almeida et al., 2009).

Our initial results suggest that RTG-dependent signaling may be activated in response to mitochondrial dysfunction in acid-stressed *S. cerevisiae* cells grown in the low pH medium used for cell pre-conditioning. In this conditions, the gene encoding peroxisomal citrate synthase *(CIT2)*, is up-regulated in ρ^0 cells compared to respiratory competent ρ^+ cells, a hallmark of RTG-dependent transcription activation. On the contrary, RTG pathway remains inactive in response to mitochondrial dysfunction in cells grown in neutral pH medium, which are sensitive to AA-PCD induction (unpublished results). This points to a possible role of RTG pathway in AA-PCD signaling (Ždralević et al., 2012).

Mitochondrial RTG signaling occurs also in mammalian cells as a result of mtDNA mutation/deletion, oxidative stress, hypoxia, treatments with specific inhibitors of the respiratory chain or drugs (Butow and Avadhani, 2004). The signaling cascade is characterized by the activation of different nuclear transcription factors, including NF-κB which controls the transcription of a variety of target genes involved in the general stress response. In terms of pro-survival and adaptive response, the RTG-dependent signaling pathway in yeast and the NF-κB pathway active in mammalian cells appear to be involved in a conserved mechanism of cell stress response (Srinivasan et al., 2010), validating yeast as a model to study mitochondrial stress response pathways (Jazwinski and Kriete, 2012; Ždralević et al., 2012).

Even a traditional industry such as wine production is taking over the challenge of tailoring genetically customized wine-yeast strains. Market-oriented wine-yeast strains are currently being developed for the cost-competitive production of wine with

minimized resource inputs, improved quality, and low environmental impact (Pretorius and Bauer, 2002). The comprehension of the complex mechanism integrating the signaling network activated by acetic acid *per se*, nutrient availability and metabolic conditions will greatly impact the improvement of both biological control of wine-spoilage microorganisms and, on the other hand, wine-yeast fermentation performances (Pretorius, 2000). With this respect, it is of note that laboratory yeast strains are unable to completely transform all the sugar in the grape must into ethanol under winemaking conditions, where multiple stresses occur simultaneously and sequentially throughout the fermentation (Mitchell et al., 2009). Post-genomic techniques and a systems biology approach will help to elucidate how the responses of wine yeasts to these stimuli differs from laboratory strains (Pizarro et al., 2007).

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Yeast adaptation to weak acids prevents futile energy expenditure

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Weak organic acids (WOAs) are widely used preservatives to prevent fungal spoilage of foods and beverages. Exposure of baker's yeast *Saccharomyces cerevisiae* to WOA leads to cellular acidification and anion accumulation. Pre-adaptation of cultures reduced the rate of acidification caused by weak acid exposure, most likely as a result of changes in plasma membrane or cell wall composition. In order to adapt to sublethal concentrations of the acids and grow, yeast cells activate ATP consuming membrane transporters to remove protons and anions. We explored to what extent ATP depletion contributes to growth inhibition in sorbic or acetic acid treated cells. Therefore, we analyzed the effect of the reduction of proton and anion pumping activity on intracellular pH (pH_i), growth, and energy status upon exposure to the hydrophilic acetic acid (HA) and the lipophilic sorbic acid (HS). ATP concentrations were dependent on the severity of the stress. Unexpectedly, we observed a stronger reduction of ATP with growth *reducing* than with growth *inhibitory* concentrations of both acids. We deduce that the not the ATP reduction caused by proton pumping, but rather the cost of sorbate anion pumping contributes to growth inhibition. A reduction of proton pumping activity may reduce ATP consumption, but the resulting decrease of pH_i affects growth more. ATP utilization was differentially regulated during moderate and severe stress conditions. We propose that the energy depletion alone is not the cause of growth inhibition during HA or HS stress. Rather, the cells appear to reduce ATP consumption in high stress conditions, likely to prevent futile cycling and maintain energy reserves for growth resumption in more favorable conditions. The mechanism for such decision making remains to be established.

Keywords: weak organic acid stress, food preservatives, intracellular pH, spoilage yeast, *Saccharomyces cerevisiae*, bioenergetics, plasma membrane pumps

INTRODUCTION

Every year, large quantities of food are contaminated by microbes and become unfit for human consumption. Food spoilage microbes in large-scale food industries is prevented among other methods with weak organic acids (WOAs) such as sorbic, acetic, benzoic, and propionic acid, which are considered to be safe for human consumption. In aqueous solution, WOAs exist in pH-dependent equilibrium between uncharged acidic molecules and their charged anions. The protonated, uncharged molecule that is abundant at low pH can freely permeate the plasma membrane. Upon encountering the high pH (∼7) inside the cell the acid dissociates to form a charged proton and anion which diffuse over the membrane anymore (Brul and Coote, 1999; Lambert and Stratford, 1999; Orij et al., 2009). Diffusion of weak acids into the cell could theoretically continue until equilibrium is reached between the concentration of the protonated state inside and outside the cell.

A first consequence of WOA stress is cellular acidification [reviewed in Piper et al. (2001), Orij et al., 2011]. Intracellular pH (pH_i) affects many cellular processes, and even a slight deviation of pH_i already affects intracellular metabolic reactions, as it influences the ionization states of acidic and basic side chains of amino acids and thereby protein activity (Orij et al., 2011). pH_i is also a critical component of the total electro-chemical gradient which is responsible for the transport of molecules across membranes (Orij et al., 2011). The release of protons and subsequent cytoplasmic acidification leads to inhibition of essential metabolic functions (Krebs et al., 1983; Bracey et al., 1998), inhibition of glycolysis (Pearce et al., 2001) and therefore reduction of the cell's ability to generate ATP. In addition, the cellular activities counteracting acidification and anion accumulation consume ATP (Piper et al., 1998; Holyoak et al., 1999). In yeast intracellular acidification is partly counteracted by the activity of Pma1p, a plasma membrane H^+-ATPase pump. *PMA1* is an essential gene that encodes the major pH_i regulator in baker's yeast (Serrano et al., 1986). It pumps H^+ ions out of the cell using ATP hydrolysis at a 1:1 ratio (de Kok et al., 2012). This activity consumes about 20% of the ATP produced during normal conditions (Morsomme et al., 2000) and up to 60% during weak acid stress (Holyoak et al., 1996). Lipophilic WOA stress also induces the plasma membrane ATP-binding cassette (ABC) transporter Pdr12p (Hatzixanthis et al., 2003), which is believed to play a role in the adaptation of *Saccharomyces cerevisiae* to these weak acids by pumping out anions (Holyoak et al., 1999) at the cost of energy, either

ATP or an aspect of membrane potential, possibly the proton gradient (Breeuwer et al., 1994; Henriques et al., 1997). *PDR12* was shown to be important for the adaptation of yeast cells to grow in the presence of lipophilic weak acid preservatives, and *pdr12*Δ mutants are hypersensitive to lipophilic acids at low pH (Holyoak et al., 1999; Hatzixanthis et al., 2003). The deletion mutant is however not sensitive to highly lipophilic, long-chain fatty acids alcohols and dicarboxylic acids (Hatzixanthis et al., 2003), and induction of the protein is not sufficient for growth in the presence of sorbic acid (Papadimitriou et al., 2007).

Once outside, the protons and anions exported from the cell will re-associate due to the low medium pH, and can diffuse back over the membrane. This constitutes a futile cycle that will provide a constant drain of the cell's energy reserves. Non-futile ATP consumption should lead to decreased diffusional entry of acid, and a decrease of pumping activity might lead to a reduced ATP burden (Cole and Keenan, 1986; Piper et al., 2001; Mira et al., 2010b). Indeed, several genome-wide analyses of the effects of WOA stress on yeast have revealed that the energy burden is significant, and that this might be a major cause of the growth inhibitory effect (Mollapour et al., 2004; Schüller et al., 2004; Mira et al., 2010a), and the effects of weak acids on yeast growth are observed as a linear relation between WOA concentration and maintenance energy requirement (Quintas et al., 2005). In this study we aimed to determine whether the accumulation of protons and anions in the cell, or rather the depletion of ATP caused by the export of both is the cause of the growth inhibitory effect of these acids.

MATERIALS AND METHODS

YEAST STRAINS AND PLASMIDS

S. cerevisiae BY4741 (*MATa his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) and the derivatives *pma1-007* (*YGL007W*::kanMX4) and *pdr12*Δ (*YPL058C*::kanMX4) were used in this study (EUROSCARF, Germany). All strains were transformed with plasmid pYES-ACT-pHluorin to measure pH_i as described earlier (Ullah et al., 2012). All chemicals were purchased from Sigma–Aldrich (Germany), unless otherwise indicated.

GROWTH CONDITIONS

Cells were grown at 30°C in a defined mineral media medium according to (Verduyn et al., 1992) using 2% glucose. Pre-inocula were grown overnight in Erlenmeyer flasks shaking at 200 rpm in the same media as above, buffered at pH 5.0 with 25 mM potassium citrate. Strains were cultivated in 500 ml batch fermenters to an initial OD_{600} of 0.1, with a steady airflow (500 ml/min) and stirring rate (600 rpm). External pH was maintained at 5.0 by titration with 0.2 M KOH using Applikon ADI 1030 Controller (Applikon, Schiedam, the Netherlands). Cultures were exposed to WOA in early exponential phase (OD_{600} ∼1.0) as indicated.

MEASUREMENT OF OD_{600} AND pH_i

pH_i was measured as described before (Orij et al., 2009). Growth (OD_{600}) and pH_i were monitored at regular intervals for 4 h. Culture samples from the batch fermenters were transferred to CELLSTAR black polystyrene clear bottom 96-well microtiter plates (BMG Labtechnologies, Germany). OD_{600} and pH_i of the cultures were measured in a FLUOstar Optima microtiter plate reader (BMG Labtechnologies, Germany) in three technical replicates.

DETERMINATION OF ACIDIFICATION RATE

Growing cultures of *S. cerevisiae* were exposed to HA (45 mM) and HS (1 mM), or control conditions at pH 5.0 and 30°C for 4 h. Cells were harvested by centrifugation at 5000 rpm for 5 min, washed, and suspended in fresh medium (with or without glucose). Pre-exposed cultures were pulsed with WOA and pH_i was monitored at one-second intervals.

NUCLEOTIDE EXTRACTION

Samples for extraction of metabolites were collected at $t = 0$, 0.5, 1, 2, 4 h after exposure of cultures to WOAs. Metabolites were extracted with boiling ethanol after quenching in methanol (Gonzalez et al., 1997). Briefly, pre-weighed cell culture samples (20 ml) were quenched using 60% (v/v) ice cold aqueous methanol (60 ml). Samples were centrifuged at 5000 g for 5 min at −20°C. Boiling ethanol 75% (v/v) was added to the pellet and incubated for 3 min at RT. After centrifugation for 5 min at −10°C the supernatant was transferred to eppendorf tubes. The liquid was evaporated using a Speed-Vac, the residue was reconstituted in 180 µl of demi water, and stored at −80°C until further analysis.

NUCLEOTIDE QUANTIFICATION

Metabolite measurements were performed by fluorimetric detection of NADH/NADPH using appropriate coupling enzymes (Gonzalez et al., 1997). Emission was measured at 460 nm after excitation at 340 nm using a FLUOstar Optima microtiter plate reader (BMG Labtechnologies, Germany).

Enzymatic determination of ATP was done at 30°C in TEA buffer (66 mM, pH 7.6) containing 6.6 mM $MgSO_4$ and 0.65 mM $NADP^+$. ATP was consumed by hexokinase (1.9 U/ml) in reaction with glucose (0.1 mM). The reaction reached an end point after 10 min, and NADH concentrations were determined.

ADP was also determined at 30°C in the buffer containing 66 mM TEA-pH 7.6, 6.6 mM $MgSO_4$, 66 mM KCl, 0.2 mM NADH, and lactate dehydrogenase (1.8 U/ml). This step eliminates pyruvate and converts it into lactate, after completion of the reaction 0.2 mM phosphoenolpyruvate and pyruvate kinase (1.8 U/ml) was added to measure the levels of ADP. The end point of this reaction was found to be 40 min after enzyme addition. The concentration of ATP and ADP was expressed in µmol/3×10^7 cells/ml assuming the number of cells at OD_{600} 1 was ∼3×10^7 cells (Orij et al., 2009). The recovery of the ATP after extraction was 90.75% and this represents the efficiency and stability of the metabolite during this extraction procedure (**Table 1**).

RESULTS

ADAPTATION TO WEAK ACIDS INCREASES PASSIVE DEFENSE MECHANISMS

S. cerevisiae acquires resistance to WOAs by activating the membrane H^+-ATPase Pma1p and transporters to expel anions. Both

processes cost energy. Baker's yeast does not degrade or metabolize the WOAs in the presence of glucose (Mollapour et al., 2008), so this energy dependent extrusion will be futile unless cells restrict the diffusional entry of WOA or increase the cellular capacity for absorption of the stress, for instance by increasing the concentration of buffering metabolites. Therefore, we probed the cells' ability to restrict the diffusional entry of weak acids. We have used two different WOAs, sorbic acid and acetic acid. Both acids have different toxic effect despite their identical pK_a values. HA inhibits growth because of its effect on cytosolic pH, whereas HS affects growth mostly by anion specific inhibitory effects (Ullah et al., 2012). We pulsed HA and HS to growing cultures of *S. cerevisiae* and estimated the initial entry by determination of the rate of intracellular acidification (**Figures 1A**,**C**). We confirmed that HS acidified cells faster than HA. Next, we pre-exposed the cultures to 45 mM of acetic acid or 1 mM sorbic acid, both of which inhibit growth by ∼50%, to induce the adaptive response. After 4 h of incubation, when the pH_i recovery is maximal [back to ∼6.5 in both cases, see Ullah et al. (2012)], cells were washed, suspended into fresh media and pulsed with 20 mM and 45 mM of HA or 1 or 1.2 mM of HS (**Figure 1**). We show only the results from the 20 mM HA pulse, as 45 mM in the absence of glucose resulted in pH_i values that were outside of the range of the calibration curve (our unpublished data). The reduction of acidification rate by the pre-exposure was, however, similar. The rate of acidification was reduced by 50%, and pre-exposure to both acids caused the cells to have a higher pH_i 1 min after an acid pulse. For HS, the results with 1 mM or 1.2 mM were similar (our unpublished data), but the reduction in entry rate was more apparent in case of the 1.2 mM pulse.

Table 1 | Metabolite recovery after extraction in boiling ethanol coupled with 60% methanol quenching.

Sample	Exogenous ATP added	Measured ATP	Difference	Recovery
1	0	8.40 ± 0.40	–	–
2	5	12.00 ± 0.80	3.60 ± 1.02	72 ± 20%
3	7.60	14.40 ± 0.36	6.00 ± 0.30	78 ± 4%
4	10	17.20 ± 1.18	8.80 ± 1.50	88 ± 15%

A reduction of the rate of acidification can be accomplished in two ways: Membranes can be altered to reduce the entry of the acids (Mira et al., 2010a), or the induction of (proton) pumping activity balancing the entry also reduces acidification (Ullah et al., 2012). To distinguish between these options, we compared the acidification of pre-exposed and control cultures of *S. cerevisiae* wild-type in the presence and absence of glucose. Removal of glucose from medium leads to the depletion of the ATP pool within minutes (Ashe et al., 2000; Thomsson et al., 2005), and thereby disables the pumping activity. Any differences in initial acidification upon WOA pulses under these circumstances should be caused by changes in influx. Although in the absence of glucose pH_i was reduced more strongly than in the presence of a source of energy, the *rate* of acidification was virtually identical in both situations (**Figure 1**). In contrast, the effect of the adaptive response

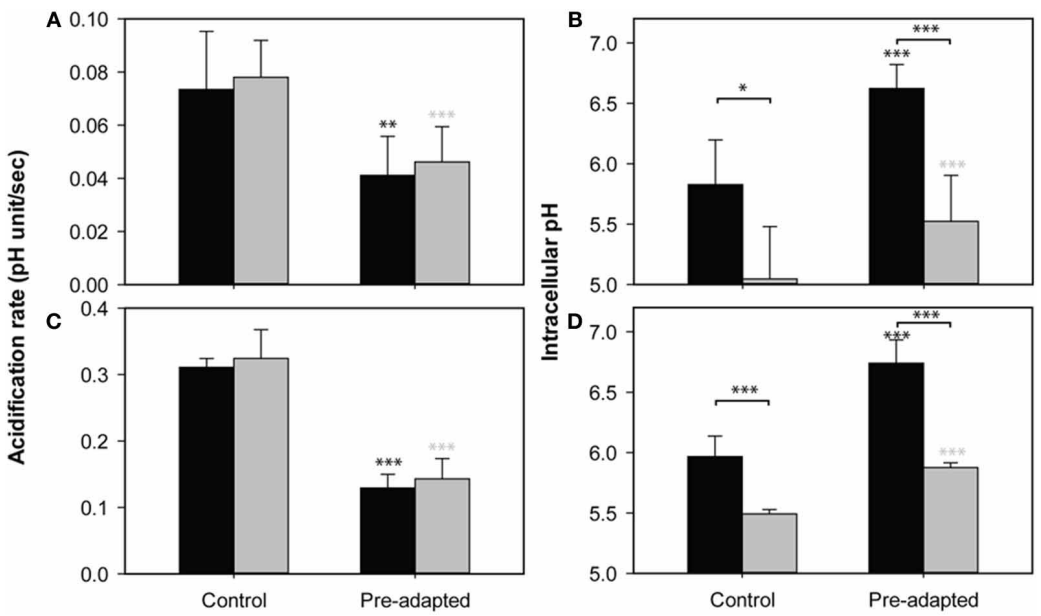

FIGURE 1 | Effect of WOA pre-exposure on acid entry. The rate of acidification **(A,C)** and pHi reduction after 1 min **(B,D)** caused by 20 mM acetic acid **(A,B)** or 1.2 mM sorbic acid **(C,D)**, in control cultures (no pre-exposure) and culture pre-exposed to 45 mM of HA **(A,B)** or 1 mM of HS **(C,D)**. The same experiment was performed in the presence (black) and absence (gray) of glucose. Data presented are the average of three independent cultivations and error bars represent standard deviations. An asterisk indicates significance of the difference between control and pre-adapted cultures, or between conditions indicated with a marker (two-tailed *t*-test; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

to WOAs induced by pre-exposure caused a decrease in the rate of acidification, which was again identical with or without glucose. This decrease in acidification rate is therefore not dependent on ATP dependent processes, and must be caused by a decreased diffusional entry of WOAs by remodeling of the cell surface. It should be noted that while the absence of an energy source did not affect the acidification rate, the pH_i reached after 1 min was lower in cultures without glucose (**Figures 1B**,**D**). Therefore, while the initial acidification rate, reflecting diffusional entry, is reduced by the pre-adaptation, adapted cells could not completely overcome the diffusional entry, and still need energy for the maintenance of pH_i in the presence of WOA. This energy demand might result in depletion of cellular ATP and therefore in reduced growth.

WOAs LEAD TO ATP DEPLETION AT LOW DOSAGES

To test whether the ATP consumption for counteracting acid stress is a major cause of growth inhibition by weak acids we measured the intracellular concentrations of adenine nucleotides (ATP, ADP), and simultaneously monitored growth and pH_i upon acid exposure. We exposed *S. cerevisiae* wild-type to concentrations of HS and HA that inhibited growth by 50 and 100% in our previous work. For acetic acid this corresponded to 40 mM and 70 mM, while for HS 1 mM and 2 mM were used. Growth and pH_i profiles are shown in **Figure 2**. Addition of WOA caused immediate acidification (**Figure 2B**). With 40 mM of HA, recovery started instantaneously, and pH_i was restored almost to the pH_i of unstressed cell after 80 min (**Figure 2B**). However, no pH_i recovery was observed with 70 mM HA stress in the time frame studied, and growth was not resumed (**Figures 2A**,**B**). Cellular ATP was depleted by 60% in 40 mM HA stressed cells. Remarkably, 70 mM caused only a 21% reduction (**Figure 2C**). ADP was also reduced in both conditions, but again more at moderate than at severe stress (**Figure 2D**).

The HS concentrations used in these conditions did not recapitulate the 60 and 90% growth inhibition observed in our previous work (Orij et al., 2009; Ullah et al., 2012), possibly due to a different experimental setup. Growth inhibition and acidification were slightly higher with 2 mM than with 1 mM HS stress. We did not observe recovery of either pH_i or growth within the time frame (**Figures 2E**,**F**).

With 1 mM HS, ATP levels decreased by ∼51% at $t = 0.5$ h and did not recover till $t = 4$ h. Decrease of ATP after 2 mM HS stress was less than after 1 mM HS (**Figure 2G**). The ADP concentrations were decreased by moderate stress conditions, while no significant decrease was observed at the higher dosage.

REDUCED ATP CONSUMPTION BY PROTON PUMPING DOES NOT IMPROVE GROWTH

We hypothesized that if ATP depletion is a stronger cause of growth inhibition than the accumulation of protons or anions itself, decreasing the pumping activity might actually improve growth. Since *PMA1* is an essential gene, we used the *pma1* hypomorph *pma1-007* (Porat et al., 2005), which has only 50% Pma1p expression and activity, to assess the effect of reduction of proton pumping associated ATP consumption on the growth inhibitory effect of sorbic and acetic acid.

Cells with reduced Pma1p activity did not show hypersensitivity to HS compared to wild-type (**Figure 3E**), and pH_i reduction and recovery were similar, consistent with our previous experiments (Ullah et al., 2012). Growth recovery of *pma1-007* coincided with pH_i restoration during HS stress. The ATP concentration initially decreased less than in wild-type, with only 28% for 1 mM, compared to 51% in wild-type. Upon 1 mM HS stress, it did however continue to decrease over the course of time. Two millimolar HS stress resulted in an ATP depletion similar to the wild-type (**Figure 3G**). In both stress conditions ADP levels were significantly reduced by the addition of the acid (**Figure 3H**). However, the reduced ATP consumption did not lead to an alleviation of the growth inhibition, suggesting that the proton extrusion coupled ATP consumption is less of a cause of growth inhibition than is the acidification itself.

We have shown previously that for HA, acidification correlates strongly with growth inhibition, and the effect of HA on growth and pH_i was enhanced by decreasing Pma1p activity (Ullah et al., 2012). Indeed, the mutant *pma1-007* was more sensitive to HA. Even moderate stress (40 mM) had a severe effect on growth of the mutant compared to wild-type (**Figure 3**). Therefore, to still assess the effect of a 50% growth inhibitory concentration, we included a 25 mM HA stress condition. Thus, three concentrations (25, 40, and 70 mM) of acetic acid stress were added to growing cultures of *pma1-007* cells. The pH_i of *pma1-007* dropped below pH 5.0 with all three concentrations, and growth and pH_i could be restored after the 25 mM HA stress only (**Figures 3A**,**B**). As observed with wild-type, the lower concentration of HA (25 mM) caused a strong depletion of ATP, while the higher concentrations (40 and 70 mM) actually led to a smaller reduction of ATP (**Figure 3C**).

Interestingly, growth of *pma1-007* was hardly affected by 25 mM HA stress, but the drop in the cellular ATP was prominent and ATP remained low up to 4 h after the initial stress (**Figure 3C**). ADP concentrations were also decreased compared to control (**Figure 3D**). Therefore, reduction of Pma1p activity did not result in higher ATP concentrations upon stress, and did not alleviate the stress phenotype. Rather, it enhanced the effect of acetic acid stress because of insufficient proton extrusion capacity. It appears, therefore, that the ATP consumption by the proton pump does not contribute strongly to the growth inhibitory effect of these two weak acids, increased acidification does.

ANION EXPULSION CONTRIBUTES STRONGLY TO ATP CONSUMPTION

The role of Pdr12p under acetic acid stress remains ill-understood. Pdr12p is not required for HA resistance and its deletion has no effect on growth in the presence of HA (Bauer et al., 2003; Ullah et al., 2012). Since Pdr12p expression is not induced by HA and its activity is not required for the extrusion of anions or protons during HA stress, we predicted that deletion of the gene would not affect adenylate nucleotide concentrations upon acetic acid stress, but would do so in case of HS exposure. Indeed, upon acetic acid stress, the growth profile of *pdr12*Δ was similar to the wild-type

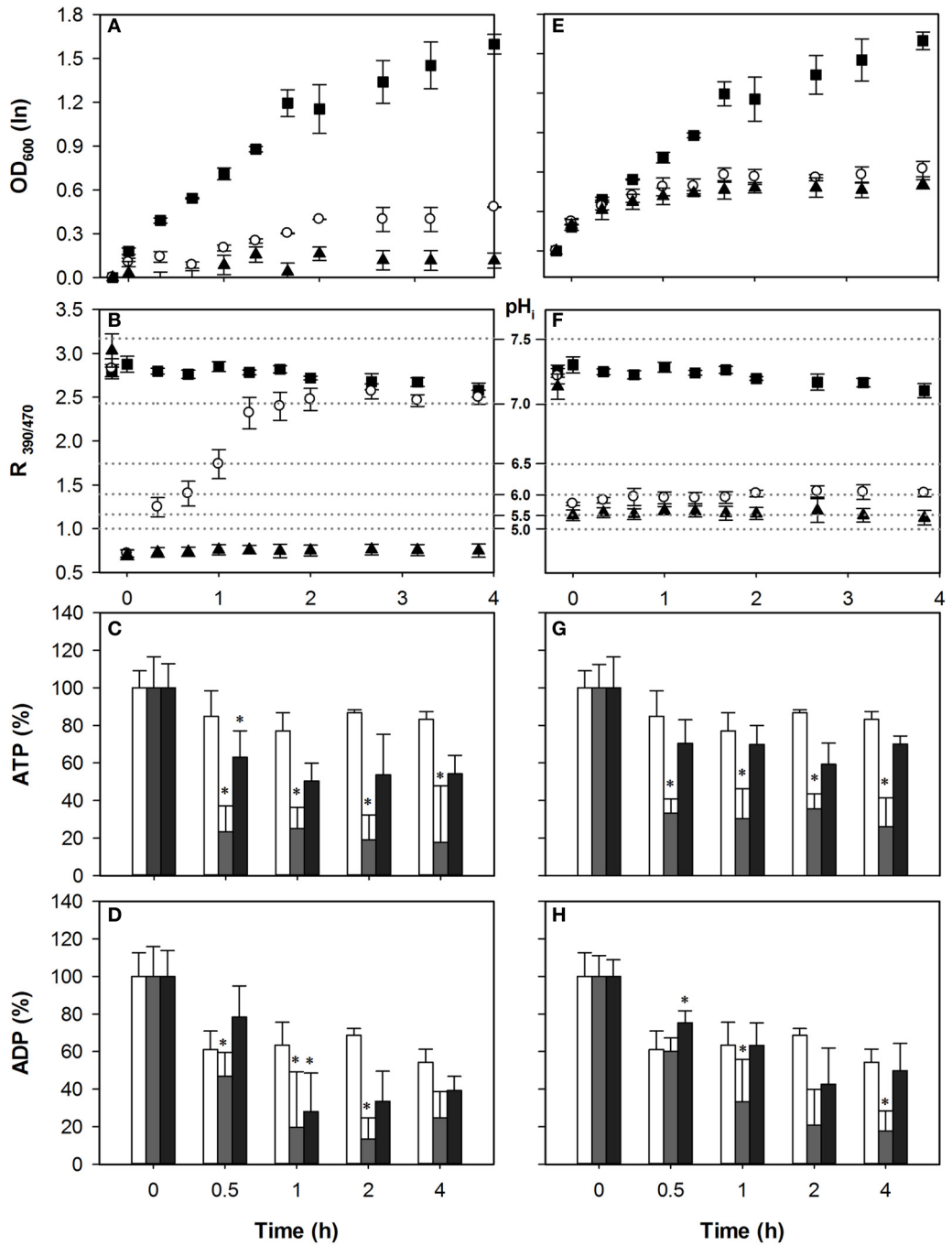

FIGURE 2 | Effect of acetic acid (left hand panels) and sorbic acid (right hand panels) on growth (A,E), intracellular pH (B,F), ATP (C,G), and ADP (D,H) levels of BY4741. Growing cultures were exposed to 0 mM (■ and white bars), 40 mM HA or 1 mM HS (○ and gray bars), and 70 mM HA or 2 mM HS (▲ and dark gray bars) at $t = 0$. pH_i data are presented as ratio of fluorescence emission upon excitation at 390 nm and 470 nm ($R_{390/470}$) while the relevant pH_i values derived from the calibration curves are also indicated. Data presented are the average of three independent cultivations and error bars represent standard deviation. An asterisk indicates significance of the difference between acid exposed and control cultures at the same time point (two-tailed *t*-test; $P < 0.05$).

(**Figure 4A**). Both concentrations led to a pH_i decrease to below 5, and as with wild-type pH_i could be restored only in the 40 mM HA stress condition (**Figure 4B**). The pattern of adenylate nucleotides observed in *pdr12*Δ was similar to that of wild-type, (**Figures 4C**,**D**) corroborating the idea that Pdr12p activity does not contribute to ATP depletion in case of acetic acid stress. Cellular ADP concentrations were also reduced (**Figure 4D**) again more strongly at lower stress.

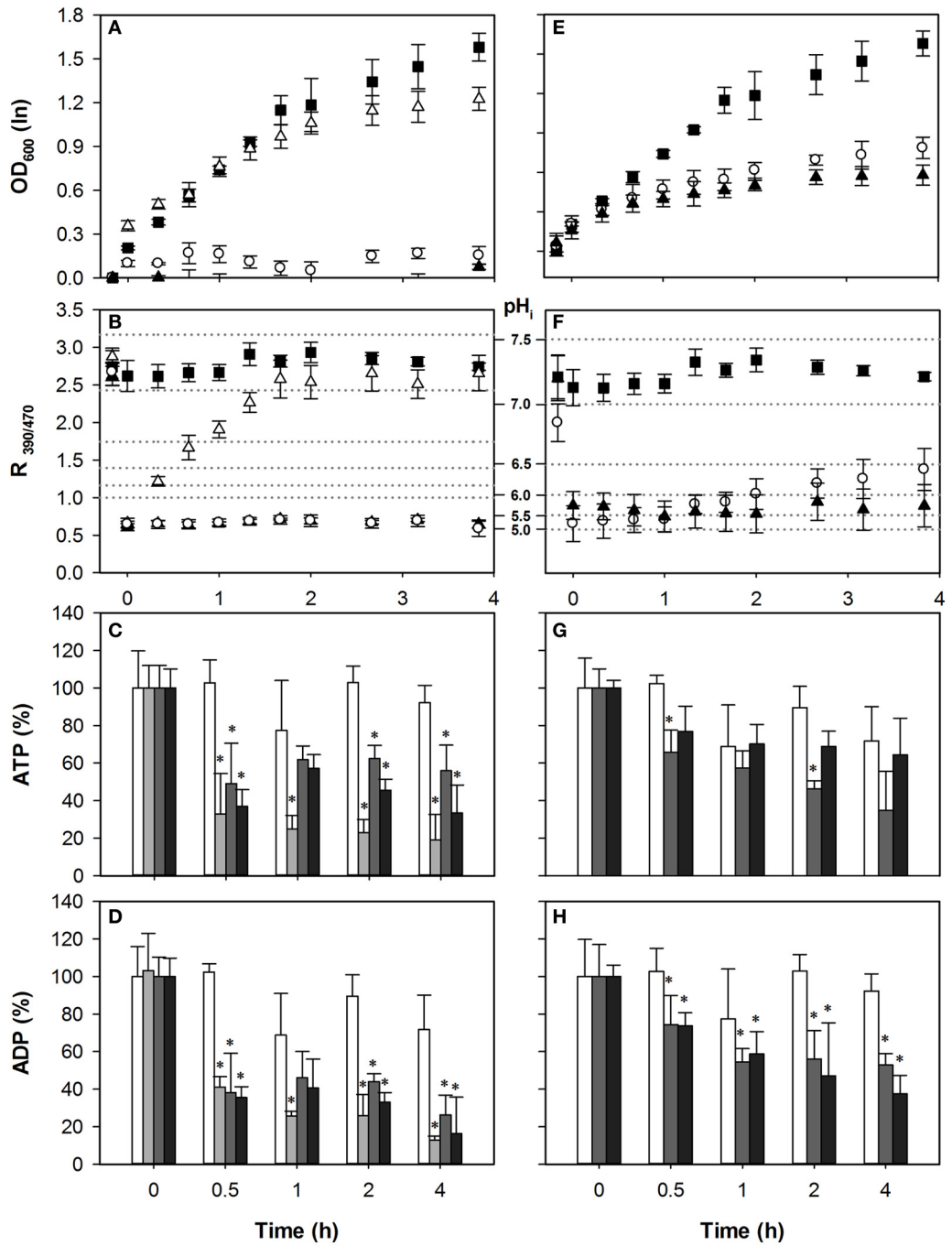

FIGURE 3 | Effect of acetic acid (left hand panels) and sorbic acid (right hand panels) on growth (A,E), intracellular pH (B,F), cellular ATP (C,G), and ADP (D,H) concentrations of *pma1-007*. Growing cultures were exposed to 0 mM (■ and white bars), 25 mM (△ and light gray bars), 40 mM HA or 1 mM HS (○ and dark gray bars), and 70 mM HA or 2 mM HS (▲ and black bars) of HA/HS at $t = 0$. pH_i data are presented as ratio of fluorescence emission upon excitation at 390 nm and 470 nm ($R_{390/470}$) while the relevant pH_i values derived from the calibration curves are also indicated. Data presented are the average of three independent cultivations and error bars represents standard deviation. An asterisk indicates significance of the difference between acid exposed and control cultures at similar time point (two-tailed *t*-test; $P < 0.05$). 40 mM HA or 1 mM HS.

Pdr12p expression is induced by sorbate and benzoate after ∼60–90 min of acid exposure to levels almost as high as Pma1p (Piper et al., 1998; Papadimitriou et al., 2007). The pH_i of *pdr12*Δ cultures decreased to 6.5 and 6.0 upon exposure to 1 mM and 2 mM of HS, respectively (**Figure 4F**). Upon exposure to 1 mM HS, *pdr12*Δ cells recovered growth after 4 h of adaptation (**Figure 4E**). However, cells treated with 2 mM HS did not recover growth and pH_i, in line with our previous analysis. If Pdr12p

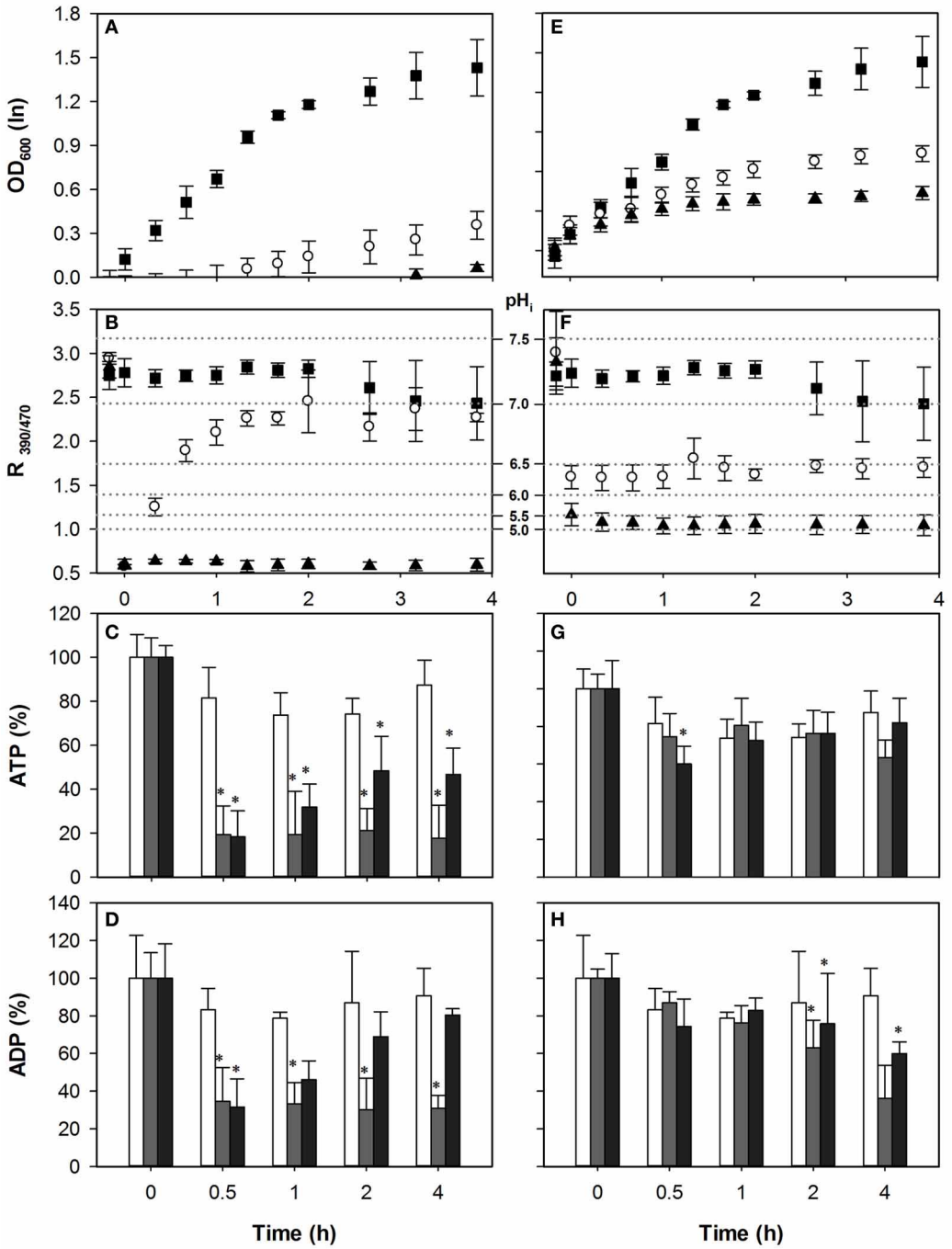

FIGURE 4 | Effect of acetic acid (left hand panels) and sorbic acid (right hand panels) on growth (A,E), intracellular pH (B,F), cellular ATP (C,G), and ADP (D,H) concentrations of *pdr12*Δ. Growing cultures were exposed to 0 mM (■ and white bars), 40 mM HA or 1 mM HS (○ and dark gray bars), and 70 mM HA or 2 mM HS (▲ and black bars) at $t = 0$. pH_i data are presented as ratio of fluorescence emission upon excitation at 390 nm and 470 nm ($R_{390/470}$) while the relevant pH_i values derived from the calibration curves are also indicated. Data presented are the average of three independent cultivations and error bars represents standard deviation. An asterisk indicates significance of the difference between acid exposed and control cultures at similar time point (two-tailed *t*-test; $P < 0.05$).

activity was a major energy consuming factor upon acid stress, we would expect that deletion of *PDR12* would reduce ATP consumption compared to wild type in the case of HS stress. Indeed, in contrast to the wild type cells, ATP levels were decreased only little during sorbic acid stress from $t = 0.5$ to $t = 4$ h as shown in **Figure 4G**. The drop in the ATP was 7% in *pdr12*Δ cells with 1 mM of sorbic acid, and 21% with 2 mM, in which case the ATP contents were mostly recovered during the

time course (**Figure 4G**). No significant difference was observed in cellular ADP concentrations during the first hour of stress exposure (**Figure 4H**).

DISCUSSION

WOAs are widely used food preservatives in food and beverage industries because they are particularly efficacious toward yeast and fungal spoilage (Winter et al., 2008). Characteristically, weak-acid preservatives do not kill yeast but rather inhibit growth (Lambert and Stratford, 1999), and this ability of yeasts to survive and proliferate in the presence of WOA is an important spoilage factor. Various mechanisms are thought to contribute to the growth inhibitory effect, but quantitative mechanistic understanding is still lacking.

We showed that the adaptation to sorbic or acetic acid resulted in a decreased diffusional entry of the molecule. It has been suggested previously that adapted cells decrease the diffusional entry of acids by remodeling their plasma membranes and reinforcement of cell wall structure to decrease its porosity (Mira et al., 2010a). Furthermore, acidification is directly affected by the extent to which the cell can buffer the protons that are released. A recent study assessed the buffering capacity of living cells, and showed this to be higher than previously estimated (Maresova et al., 2010). However, this analysis of buffer capacity determined not only the passive buffering, by proton association to intracellular weak acids, but a combination of this passive buffering with active extrusion of protons to counteract the diffusional entry (Mollapour et al., 2008). To distinguish between the passive contributions of altered membrane composition and/or intracellular buffer capacity, and active proton efflux, we analyzed how the initial acidification rate depended on both of these aspects. Because the rate of acidification was not dependent on the presence of an energy source it reflects acid entry. Therefore, we concluded that pre-exposed cells indeed decrease the entry by alteration of either the structure of the plasma membrane or their cell wall composition, or by increasing intracellular buffering. Additionally, pH *recovery* depended strongly on the presence of an energy source.

EFFECT OF WOA ON THE CELLULAR CONCENTRATION OF ATP

The intracellular concentration of ATP depends on the balance between energy production and consumption. Acetic and sorbic acid are known to stimulate different response in yeast, but in both stress conditions *S. cerevisiae* uses energy dependent membrane transporters to expel protons and anions, which may deplete the cellular ATP pool and thus inhibit growth (Breeuwer et al., 1994; Holyoak et al., 1999; Mollapour et al., 2008). HS was previously shown not to affect ATP production (Holyoak et al., 1996). Therefore we assume that a decrease in ATP observed is due to increased consumption, likely because of the activities of Pma1p and Pdr12p, the major proton and anion exporter during HS stress in *S. cerevisiae* (Morsomme et al., 2000; Mollapour et al., 2008). We showed that cells do use ATP in the presence of WOA but its depletion is not a major contribution to growth inhibition in the case of acetic acid: If ATP depletion would be the major cause of growth inhibition, then reduction of ATP dependent extrusion of WOA protons and anions should increase cellular ATP, and this should then relieve the growth inhibitory effect of the WOA compared to the drop in pH. Decreased expression of Pma1p in the *pma1-007* mutant slightly reduced ATP consumption upon in response to HA exposure, but the mutant was more sensitive rather than less (Stratford et al., 2009; Ullah et al., 2012). This increased susceptibility of *pma1-007* is likely due to the increased acidification rather than ATP consumption. As expected, growth, pH_i and intracellular ATP levels in *pdr12*Δ cells were similar to the wild-type cells, reconfirming that Pdr12p is not instrumental in HA tolerance (Mollapour et al., 2008). ATP consumption for proton expulsion is not a major cause of growth inhibition in case of HS stress either: Reduction of ATP consumption by Pma1p indeed resulted in increased ATP concentrations, but this did not alleviate the growth inhibitory effect of the acid. Interestingly, deletion of *PDR12* also reduced ATP consumption upon sorbic acid exposure, and this now correlated with improved growth behavior [as observed before Ullah et al. (2012)]. This reconfirms that Pdr12p is active in response to HS (Holyoak et al., 1996, 1999; Piper et al., 1998), and suggests that its activity is indeed a significant ATP burden. Therefore, Pma1p and Pdr12p are active during HS stress, but neither the proton pumping nor the anion expulsion activities is truly key for adapted growth in the timeframe observed in the presence of these low dosages of sorbic acid in *S. cerevisiae* (Papadimitriou et al., 2007; Ullah et al., 2012). Because the growth inhibition phenotype of sorbic acid was slightly alleviated in the *pdr12*Δ mutant, we conclude that the ATP consumption by Pdr12p is a larger burden to cellular functioning than is the sorbate anion accumulation.

Overall, we observed that when cells were subjected to severe WOA stress, they reduced the ATP expenditure for the recovery of either growth or pH_i. This suggests that yeast has evolved mechanisms that prevent WOA induced ATP depletion, since ATP levels were higher in severe stress conditions where growth was completely inhibited, compared to moderate stress where ATP levels were lower but growth was partially inhibited. This phenomenon was seen with both acetic acid, where we have shown previously that growth inhibition was mainly caused by acidification (Ullah et al., 2012), and with the more lipophilic sorbic acid, where we expected other aspects of WOA to lead to growth inhibition. This implies that cells somehow sense the severity of the stress and change the strategy of adaptation, not consuming ATP for futile attempts at recovery, but rather reserving it, in spite of the low pH_i and high anion concentrations that now necessarily persist in the cell. Indeed, the H^+-ATPase was shown to be inhibited by a chaperone, Hsp30p, which is induced upon stress (Piper et al., 1997). This should then be advantageous for later recovery of growth. Interestingly, we have recently shown that yeast encounters similarly low pH_i values every growth cycle, since upon glucose depletion, the cytosolic pH eventually drops to environmental pH (Orij et al., 2012), without consequences for cell viability in the course of several days. Because a low pH_i is a (proxy of a) signal for nutrient depletion or pending energy limitation (Dechant et al., 2010; Young et al., 2010; Orij et al., 2012), it is tempting to speculate that this signal could be used for safeguarding proper energy homeostasis or distribution upon nutrient depletion, to ensure stationary phase survival (Thomsson et al., 2005). Indeed, addition of glucose causes rapid

phosphorylation of Pma1p, leading to its activation (Lecchi et al., 2007). This fits the notions of the interaction of nutrient sensing and growth control in response to environmental stress, where optimization of efficiency was also shown to be of key importance (Vilaprinyo et al., 2010; Zakrzewska et al., 2011). Understanding how pH_i affects cellular decision making to control the biochemical activity could provide powerful tools in understanding the biological mechanism of stress response and adaptation.

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Growth and membrane fluidity of food-borne pathogen *Listeria monocytogenes* in the presence of weak acid preservatives and hydrochloric acid

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This study addresses a major issue in microbial food safety, the elucidation of correlations between acid stress and changes in membrane fluidity of the pathogen *Listeria monocytogenes*. In order to assess the possible role that membrane fluidity changes play in *L. monocytogenes* tolerance to antimicrobial acids (acetic, lactic, hydrochloric acid at low pH or benzoic acid at neutral pH), the growth of the bacterium and the gel-to-liquid crystalline transition temperature point (T_m) of cellular lipids of each adapted culture was measured and compared with unexposed cells. The T_m of extracted lipids was measured by differential scanning calorimetry. A trend of increasing T_m values but not of equal extent was observed upon acid tolerance for all samples and this increase is not directly proportional to each acid antibacterial action. The smallest increase in T_m value was observed in the presence of lactic acid, which presented the highest antibacterial action. In the presence of acids with high antibacterial action such as acetic, hydrochloric acid or low antibacterial action such as benzoic acid, increased T_m values were measured. The T_m changes of lipids were also correlated with our previous data about fatty acid changes to acid adaptation. The results imply that the fatty acid changes are not the sole adaptation mechanism for decreased membrane fluidity (increased T_m). Therefore, this study indicates the importance of conducting an in-depth structural study on how acids commonly used in food systems affect the composition of individual cellular membrane lipid molecules.

Keywords: *Listeria monocytogenes*, membrane fluidity, phase transition, DSC, acid stress response, preservatives, weak acids, hydrochloric acid

INTRODUCTION

Listeria monocytogenes has been associated with a variety of food products, including dairy foods, meat, poultry, and seafood as well as fruits and vegetables (Farber and Peterkin, 2000; Mastronicolis et al., 2011). In 2008, 1,381 confirmed human cases of listeriosis were reported in the European Union and the reported case-fatality rate was 20.5% [European Food Safety Authority (EFSA), 2010].

Modification of membrane lipid composition is clearly an important adaptation mechanism in *L. monocytogenes*, which allows it to grow in a stressful environment such as low temperature (Annous et al., 1997; Mastronicolis et al., 2005); low pH (Giotis et al., 2007; Mastronicolis et al., 2010); presence of disinfectants (Bisbiroulas et al., 2011); pressure; ion concentrations etc. (Beales, 2004). Changes in lipid composition can lead to changes in cytoplasmic membrane fluidity (Mykytczuk et al., 2007).

The term "membrane fluidity" is a convenient one to summarize a multifaceted phenomenon that has contributions from molecular packing (order) and molecular motions (viscosity; Russell, 2002). Membranes can exist in different phases and the most consistent phase transition is the one occurring when the membrane passes from a tightly ordered "gel" or "solid" phase to a liquid-crystal phase which is the active state of the membrane. A widely used method for determining the phase transition temperature (T_m) is calorimetry. The influence of hydrocarbon chain length, branching and unsaturation, as well as the head group of the membrane lipids on the value of T_m, is considerable. In general, increasing the chain length, decreasing the branching or increasing the saturation of the chains increases the phase transition temperature (New, 1994; Mykytczuk et al., 2007).

Weak lipophilic acids can occur naturally in many fruits and vegetables and have been widely used to maintain microbial stability in low pH foods. Weak acid preservatives affect the cells' ability to maintain pH homeostasis, disrupting substrate transport and inhibiting metabolic pathways (Beales, 2004). The effect of many weak acid preservatives is dependent on the fluidity and permeability of the cytoplasmic membrane, since it is the first barrier to encounter the stress and any sensing mechanism would be located within it (Beales, 2004; López et al., 2006). Changes in the lipid profile of the plasma membrane may alter membrane permeability and fluidity, which may in turn contribute to tolerance (Beales, 2004).

In our previous report on the effects of different acidic stresses such as hydrochloric, acetic, and lactic acid (pH 5.5) or benzoic

acid (pH 7.3) on *L. monocytogenes* total, polar and neutral lipid compositional changes, our results suggest that only low pH value enhances the antimicrobial activity of an acid, though irrespective of pH, the acid adaptation response leads to a similar alteration in fatty acid composition, mainly originating from the neutral lipid class of adapted cultures (Mastronicolis et al., 2010). However, the effects of the aforementioned acidic antimicrobials on membrane fluidity in *L. monocytogenes* have not been determined and compared to date. The present work was intended to provide new data by determining and comparing modifications in T_m of *L. monocytogenes* membrane lipids (and thus alterations in membrane fluidity) in response to acid stress induced by acids such as hydrochloric, acetic, lactic, or benzoic acids and also to correlate the fatty acid compositional changes of each acid-adapted culture (from our previous data) with the lipid thermodynamic behavior in order to clarify if modifications in the membrane physical state of adapted cells act as a defense mechanism against acid stress.

MATERIALS AND METHODS

CULTURE OF THE ORGANISM

An avirulent strain *L. monocytogenes*, DP-L1044 (D. Portnoy, University of Pennsylvania) prepared by a transposon insertion (Camilli et al., 1991) in the parent strain (Lm10403S), was grown in brain heart infusion broth (BHI, Difco Laboratories) at 30°C (24 h). A 10 mL aliquot of this was then inoculated into 1 L of BHI broth, which was then incubated at 30°C ($Lm_{control}$) until early stationary phase. Four aliquots (10 mL) of the same stock were then inoculated, respectively, into 1 L BHI that were adjusted to $pH_{initial}$ 5.5 with (i) HCl (Lm_{HCl}); (ii) L-lactic acid (Fluka, PA, USA; Lm_{LA}); and (iii) acetic acid (Merck, PA, USA; Lm_{AA}). Another 10 mL aliquot was used to inoculate 1 L BHI with the addition of 1.00 g benzoic acid (Merck, PA, USA; Lm_{BA}) $pH_{initial}$ 7.3. All the above cultures were incubated at 30°C until early stationary phase. The growth of *L. monocytogenes* for each treatment over time was determined by measuring absorbance (OD) at 600 nm.

EXTRACTION OF TOTAL LIPIDS

From each culture, cells pelleted by centrifugation (4°C, 5877 × *g*) were washed twice in phosphate buffer (pH 7.0). Extraction of total lipids performed essentially by extraction with chloroform/methanol (2/1 v/v) and washing the extract with 0.2 volumes of water (Folch et al., 1957). After phase equilibration, the lower chloroform layer (total lipids) was dried under nitrogen.

DIFFERENTIAL SCANNING CALORIMETRY ANALYSIS

Two sets of extracted total lipids from each acid-adapted or non-adapted culture were utilized for differential scanning calorimetry (DSC) analysis. Each set of extracted total lipids was collected from one culture, in the case of $Lm_{control}$ and of Lm_{BA}, or by harvesting two cultures in the case of Lm_{AA} and Lm_{HCl}, in order to obtain the appropriate weight of lipids for DSC analysis (4–5 mg). Notably, in the case of Lm_{LA}, one set of extracted total lipids was used because the appropriate weight of lipids for DSC analysis was collected by harvesting five cultures.

Portions of the samples (approximately 4 mg) were weighed in stainless-steel capsules obtained from PerkinElmer (Norwalk, CT, USA) and sealed. Thermal scans were obtained using a PerkinElmer DSC-7 calorimeter and Pyris software for Windows. All samples were scanned from −25 to 80°C until identical thermograms were obtained, using a scanning rate of 10°C min^{-1}. The temperature scale of the calorimeter was calibrated using indium (T_m = 156.6°C) and dipalmitoylphosphatidylcholine from Avanti Polar Lipids Inc. (Alabaster, AL, USA) bilayers (T_m = 41.2°C). The following diagnostic parameters in the observed endothermic events were recorded during the phase transition and are used for the study of lipids: T_m (maximum of the temperature peak), and ΔH (the area under the peak represents the enthalpy change during the transition).

The repeatability of the thermograms and reversibility of the transitions were checked after each run by re-heating the sample after cooling. All samples were scanned a minimum of three times.

STATISTICAL ANALYSIS

The results were evaluated by analysis of variance (ANOVA). *T*-test for unpaired observations was tested at a confidence level of 95%.

RESULTS

Growth of *L. monocytogenes* in BHI medium with time was determined for each treatment by measuring absorbance (OD) at 600 nm and shown in **Figure 1**. The presence of lactic, acetic, or hydrochloric acid at pH 5.5 was accompanied by low survival ($P < 0.01$), while cells grown at neutral pH in the presence of benzoic acid displayed little antilisterial activity ($P < 0.05$). The obtained OD_{600} values were at early stationary phase: $Lm_{control}$ 0.811 ± 0.010, 10 h; Lm_{LA} 0.096 ± 0.018, 168 h; Lm_{AA} 0.217 ± 0.019, 72 h; Lm_{HCl} 0.320 ± 0.014, 24 h; and Lm_{BA} 0.694 ± 0.019, 10 h.

$Lm_{control}$ CELLS

The DSC analysis revealed T_m value 25.78 ± 1.06°C as well as enthalpy difference (ΔH) 8.99 ± 0.557 J g^{-1} (**Table 1** and **Figure 2**).

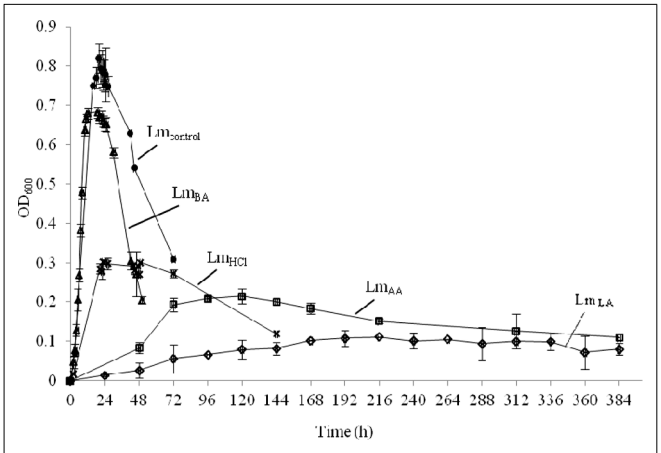

FIGURE 1 | Growth of *L. monocytogenes* before (•, $Lm_{control}$) and after acid stress exposure by lactic (◇, Lm_{LA}), acetic (□, Lm_{AA}), hydrochloric (×, Lm_{HCl}), or benzoic (△, Lm_{BA}) acid.

Table 1 | Data from differential scanning calorimetry analysis of *L. monocytogenes* total lipids before ($Lm_{control}$) and after acid stress exposure by lactic (Lm_{LA})[d], acetic (Lm_{AA}), hydrochloric (Lm_{HCl}), or benzoic (Lm_{BA}) acid.

	$Lm_{control}$	Lm_{AA}	Lm_{HCl}	Lm_{BA}
T_m (°C)	25.78 ± 1.06	29.35 ± 0.23[a] (T_{m1})	29.23 ± 0.21[a] (T_{m1})	30.25 ± 2.01[a]
		34.72 ± 2.28[a] (T_{m2})	32.28 ± 0.56[a] (T_{m2})	
ΔH (J g^{-1})	8.990 ± 0.557	14.921 ± 0.168[b]	8.246 ± 0.178	11.618 ± 0.401[a]
ΣBCFA/ΣSSCFA[c]	8.3	1.6	2.1	2.6

T_m, phase transition temperature; ΔH, enthalpy difference.
[a]*Values statistically increased compared to $Lm_{control}$, $P < 0.05$.*
[b]*Values statistically increased compared to $Lm_{control}$, $P < 0.01$.*
[c]*Ratio of total branched-chain fatty acids, BCFA, to total saturated straight chain fatty acids, SSCFA, of total lipid fatty acid profiles of cells. These data were derived from our previous study (Mastronicolis et al., 2010).*
[d]*The data for Lm_{LA} were: 27.83° C for T_m, 7.984 J g^{-1} for ΔH, and 1.4 for ΣBCFA/ΣSSCFA. One set of extracted lipids was utilized because the appropriate weight of lipids for DSC analysis was collected by harvesting five cultures.*

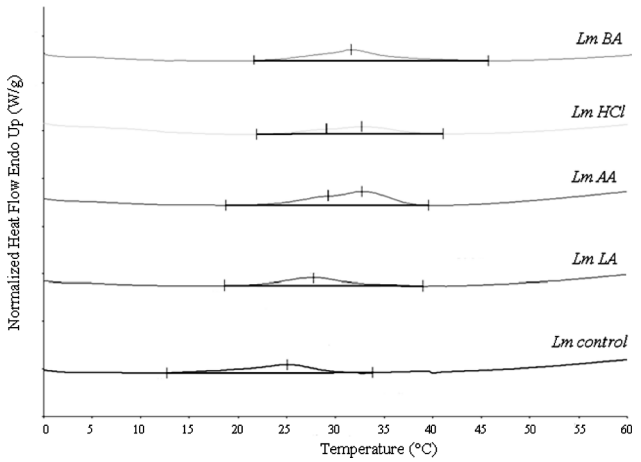

FIGURE 2 | Differential scanning calorimetry curves of *L. monocytogenes* total lipids, before ($Lm_{control}$) and after acid stress exposure by lactic (Lm_{LA}), acetic (Lm_{AA}), hydrochloric (Lm_{HCl}), or benzoic (Lm_{BA}) acid.

Lm_{AA} AND Lm_{HCl} CELLS

The DSC analysis of each sample revealed two distinct peaks of increased T_m values (T_{m1} and T_{m2}) compared to $Lm_{control}$. The Lm_{AA} sample showed differences of +3.57 and +8.94°C for T_{m1} and T_{m2}, respectively ($P < 0.05$), also Lm_{HCl} sample showed differences of +3.45 and +6.50°C, respectively ($P < 0.05$).

Lm_{LA} CELLS

In the DSC analysis an increased T_m value was measured, in which the difference was +2.05°C higher than $Lm_{control}$.

Lm_{BA} CELLS

In the DSC analysis an increased T_m value was measured, in which the difference was +4.47°C higher than $Lm_{control}$ ($P < 0.05$).

As concerns the ΔH values for each instance of acid-adapted cells, the observed changes were as follows: Lm_{AA}: 66% ($P < 0.01$), Lm_{BA}: 29.2% ($P < 0.05$), increase compared to $Lm_{control}$. For the rest samples the ΔH values were similar to $Lm_{control}$ (**Table 1**).

DISCUSSION

Other authors examined the antilisterial effects of these acids. Ravichandran et al. (2011) observed that benzoic acid (5 g/L) demonstrated antimicrobial activity against *L. monocytogenes* after 72 h incubation at 37°C. Heavin et al. (2009) observed that benzoic acid was more effective at inhibiting growth of *L. monocytogenes* than acetic acid, in a medium with a pH of 6.4 (acidified with HCl). Hydrochloric, lactic, and acetic acids at pH 3.5 gave similar kill curves (O'Driscoll et al., 1996). Hydrochloric acid caused low survival of *L. monocytogenes* at pH 5 (Karatzas et al., 2010) and slight antibacterial action against *L. monocytogenes* was observed with acetic acid at pH 5 (Chavant et al., 2004). In contrast, Vasseur et al. (1999) observed that the antilisterial effect was: acetic acid > lactic acid > hydrochloric acid. Similar results were observed by Bonnet and Montville (2005) in *L. monocytogenes* growing at pH 3.5. Phan-Thanh et al. (2000) also found that acetic acid had a more deleterious effect on *L. monocytogenes* than hydrochloric acid did. Exposure to lactic acid at pH 4.0 totally inactivated *L. monocytogenes*, whereas exposure at pH 4.5 had inhibitory effect (at 5 or 10°C), therefore, even small differences in pH, such as 0.5 units, may have a major impact on the survival of pathogens and hence, on food safety (Tiganitas et al., 2009). The comparative study of acid habituation of *L. monocytogenes*, under the same experimental conditions is important for the identification of differences between the survival of the pathogen, as comparison between laboratories is difficult because of variation in the assay conditions used (exact pH value, bacterial strains, incubation temperatures, etc.).

This study provides a first approach to observing the role of phase transitions of membrane lipids (membrane fluidity) in the acid adaptation response of *L. monocytogenes*. We have previously studied the lipid composition of *L. monocytogenes* cells grown in the presence of various acids (hydrochloric, acetic, lactic, and benzoic acid) and the analysis of membrane lipids revealed that *L. monocytogenes* similarly altered its fatty acid composition by incorporation more straight (mostly $C_{16:0}$, $C_{18:0}$,and $C_{14:0}$) and fewer branched-chain fatty acids into its membrane independently of the acid utilized (**Table 1**; Mastronicolis et al., 2010). It is expected that these fatty acids changes lead to membranes with decreased fluidity and low permeability properties (Kaneda, 1991; Zhang and Rock, 2008). In the current study, the measured lipid T_m value of

each set of adapted cells was increased compared to $Lm_{control}$ and this observation is interpreted by the above fatty acid compositional changes. However, the increases in T_m values are not of equal extent and therefore are not absolutely reflected by the acyl chain compositional changes. This fact indicates that fatty acid changes may be crucial but they are not the sole mechanism by which *L. monocytogenes* perceives the acid stress (alters its membrane lipids). Furthermore, the growth of *L. monocytogenes* in the presence of hydrochloric, lactic, and acetic acid at pH 5.5 caused an increase of neutral lipid percentages (Mastronicolis et al., 2010).

Hydrochloric acid will be dissociated, whereas acetic ($pK_a = 4.74$) and lactic acid ($pK_a = 3.79$) will be undissociated at pH 5.5. The latter form of both organic acids is membrane-permeable and thus allows acetic and lactic acid to enter the microbial cell. In this work, when the cells were grown in the presence of acetic or hydrochloric acid, the highest T_m values and low survival were observed (**Figure 1**; **Table 1**), suggesting that the decrease in membrane fluidity was related to low survival. However, this tendency was reversed in the case of lactic acid, which caused the highest antimicrobial action (**Figure 1**) in *L. monocytogenes* cells and these data cannot be explained by a modification in membrane fluidity, which was minimal. This suggests that the membrane fluidity can serve only as a preliminary tool to make predictions concerning the viability of cells. Also, another interesting point was that acetic and hydrochloric acid caused two distinct phase transition points: lipids with different fatty acyls as well as different head groups, whose T_m values differ greatly from each other, undergoing phase transitions independently, and forming membranes composed of two or more separate phases. If the fatty acyls or the head groups have similar T_m values, a main transition intermediate in temperature between those of the individual components will be given (New, 1994). Mykytczuk et al. (2010) also observed decreases in membrane fluidity along with two distinct phase transition points in some strains of *Acidithiobacillus ferrooxidans* in sub-optimal pH.

Benzoic acid ($pK_a = 4.19$) at pH 7 will be in its dissociated form (benzoic anion) and this form is less membrane-permeable and thus does not facilitate its entrance to the microbial cell. The used amount of benzoic acid (1 g/L culture) did not reduce the pH of the medium. In order to reduce the pH value, even more amount of benzoic acid might be added (that is inappropriate for food systems) or one more acid should be added along to benzoic acid (that it is out of the aim of the current work, which was the study of each acid separately). Unlike the rest of the acids utilized, in the presence of benzoic acid the percentage of neutral lipid class remains constant but the decrease of negatively charged phospholipids, such as cardiolipin or phosphatidylglycerol (Mastronicolis et al., 2010), leads to a decrease in membrane fluidity, i.e., increased T_m value (New, 1994), and the data of the present study are consistent with this increase in T_m. Furthermore, high T_m value and low antibacterial action (**Figure 1**; **Table 1**) was observed, suggesting that the decrease in membrane fluidity was related to the low antibacterial activity of benzoic acid. The low antibacterial action of benzoic acid might be arisen from the neutral pH of the medium. Relevant to our current work in the case of benzoic acid, Alonso-Hernando et al. (2010) also observed that decreased membrane fluidity in *L. monocytogenes* was correlated to survival upon acid stress, suggesting that adaptation to acid decontaminants is related to changes in membrane fluidity.

Listeria monocytogenes and *Salmonella enterica* cells exposed to sub-inhibitory concentrations of acid decontaminants (citric acid and peroxyacids) showed decreased membrane fluidity (Alonso-Hernando et al., 2010). In sub-optimal pH, a decrease in membrane fluidity of *A. ferrooxidans* was observed and this is likely linked to the overall increase in saturated fatty acids at the expense of unsaturated fatty acids (Mykytczuk et al., 2010). Adaptation to acid and starvation stress increased net cell hydrophobicity and decreased membrane fluidity of *L. innocua* (Moorman et al., 2008). ATR(+) *L. monocytogenes* cells [cells exposed to mild acid (pH 5.5), which are subsequently able to resist severe acid (pH 3.5) conditions] had lower membrane rigidities than ATR(−) cells (cells subjected at pH 3.5 directly; Najjar et al., 2009). After exposure to oregano essential oil concentrations up to 0.50%, the membrane fluidity of *L. monocytogenes* was decreased presumably to block, or at least to reduce essential oil entrance and partition into the membrane (Serio et al., 2010). Growth in the presence of butyrate, leucine, valine, isovalerate, or isobutyrate increased the calculated (theoretical estimation) transition temperature of *L. monocytogenes* cells, because of the decrease of branched-chain at the expense of saturated-chain fatty acids (Julotok et al., 2010). Increase in phase transition temperatures was observed with increased osmotic pressure in *Saccharomyces cerevisiae* (Laroche et al., 2001). Decreased membrane fluidity was also observed in *Bacillus subtilis* subjected to osmotic pressure (López et al., 2006).

An understanding of phase transitions and fluidity of membranes is important; since the phase behavior of a membrane determines such properties as permeability, fusion, aggregation, and protein binding, affects critical biochemical reactions, transport systems, all of which can markedly affect the stability of membranes, and their behavior in the cell (New, 1994; Yuk and Marshall, 2006). Acid habituation of pathogens may enhance survival in an acidic food or in the stomach and subsequently cause infection after ingestion. The resistance or adaptation of pathogens to such conditions affect food safety and thus is clearly of significance to the food industry (Beales, 2004).

Although the acid adaptation response of *L. monocytogenes* altered the fatty acid composition similarly, irrespective of the acid utilized (Mastronicolis et al., 2010), in the present study observed T_m values were increased but not equally. This suggests that the T_m value (membrane fluidity) of lipids does not depend only on the acyl constituent, but also on the total composition and nature of the lipid molecular structure (e.g., phospho-, glyco-, amino-head groups for polar lipids or the specific lipid molecule for neutral lipids, e.g., diclycerides, esters, waxes, etc.). Thus, understanding the physical chemistry of membrane lipids is important in the sense that the characteristics of lipid species, and their heterogeneity, all affect biological membranes. Our current understanding of the role of individual lipid species in a heterogeneous lipid matrix and the specific lipid–lipid and lipid–protein interactions is still far from comprehensive. Therefore, one conclusion of this study would support the in-depth identification of the membrane polar and neutral lipid molecules of *L. monocytogenes* cells in the presence of the acids utilized. Furthermore, in this study an avirulent

mutant strain was used. Previous studies have revealed that this strain has similar fatty acid composition as wild strains in optimal condition of growth or in cold adaptation (Annous et al., 1997; Mastronicolis et al., 1998, 2010; Chihib et al., 2003; Julotok et al., 2010), thus we suppose that this mutation will have no impact on the present results response to acids. However, more studies may be required with more strains in order these results to be confirmed because there are not sufficient studies in this field.

In conclusion, in this study we observed that adaptive response of *L. monocytogenes* to weak or strong acid food preservatives includes an increase in the total lipid T_m (decreased membrane fluidity), decreasing the ability of the weak acid preservatives to pass through the membrane and to act into the microbial cell, and thus conferring protection. Furthermore, decreased membrane fluidity acts as strong defense mechanism in some conditions (in the cases of hydrochloric or acetic acid) or as mild defense mechanism (in the cases of benzoic or lactic acid).

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Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium*

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Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host. Most of the probiotic bacteria currently available in the market belong to the genera *Lactobacillus* and *Bifidobacterium*, and specific health-promoting activities, such as treatment of diarrhea or amelioration of gastrointestinal discomfort, have been attributed to them. In order to be able to survive the gastrointestinal transit and transiently colonize our gut, these bacteria must be able to counteract the deleterious action of bile salts, which are the main components of bile. Bile salts are detergent-like biological substances synthesized in the liver from cholesterol. Host enzymes conjugate the newly synthesized free bile acids in the liver with the amino acids glycine or taurine, generating conjugated bile salts. These compounds are stored in the gall bladder and they are released into the duodenum during digestion to perform their physiological function, which is the solubilization of fat coming from diet. These bile salts possess strong antimicrobial activity, since they are able to disorganize the structure of the cell membrane, as well as trigger DNA damage. This means that bacteria inhabiting our intestinal tract must have intrinsic resistance mechanisms to cope with bile salts. To do that, *Lactobacillus* and *Bifidobacterium* display a variety of proteins devoted to the efflux of bile salts or protons, to modify sugar metabolism or to prevent protein misfolding. In this manuscript, we review and discuss specific bile resistance mechanisms, as well as the processes responsible for the adaptation of bifidobacteria and lactobacilli to bile.

Keywords: *Lactobacillus, Bifidobacterium*, bile resistance, bile adaptation, bile detoxification

INTRODUCTION

Strains of *Lactobacillus* and *Bifidobacterium* have been extensively used as probiotic microorganisms for humans (Sánchez et al., 2012). In order to reach the colon in a viable state, they must cope with specific stress challenges throughout the gastrointestinal tract, among which the presence of bile in the upper parts of the small intestine is one of the main ones. The main components of bile are bile acids, which are produced and conjugated with the amino acids glycine or taurine in the liver, to generate conjugated bile salts (Hofmann, 1994). Bile is stored in the gall bladder and flows from there to the duodenum during digestion, facilitating the solubilization and absorption of dietary fats. Thus, under normal physiological conditions, our intestine holds a bile salt concentration gradient ranging from more than 40 mM to less than 1 mM – equivalent to a range between 2% and 0.05% – which is responsible, among other factors, for shaping the microbial community profile found in our gut (Islam et al., 2011).

Apart from its normal physiological function, bile is highly toxic for those microorganisms unadapted to the intestinal conditions. Therefore, enteric bacteria, including lactobacilli and bifidobacteria, must have evolved specific defense mechanisms to resist the deleterious action caused by these compounds. The strong lipophilic nature of the steroid ring makes the cell membrane the main target of these molecules, in which they disturb the lipid packaging and disrupt the proton motive force, causing cell death (Kurdi et al., 2006). Furthermore, since the unconjugated forms are weak acids, they can passively diffuse into the cell and, once inside, they are dissociated producing an acidification of the cytoplasm (Sánchez et al., 2013). Other side effects induced by bile have been documented, including induction of oxidative stress and DNA repair mechanisms, alterations of sugar metabolism, and protein misfolding (Begley et al., 2005). Thus, in this review we would like to summarize the current knowledge on the mechanisms used by lactobacilli and bifidobacteria to counteract the effect of bile acids on cell physiology.

COMMON ASPECTS OF BILE RESISTANCE MECHANISMS IN *Lactobacillus* AND *Bifidobacterium*

Bile tolerance is one of the most crucial properties for probiotic bacteria, as it determines its ability to survive in the small intestine, and consequently its capacity to play its functional role as a probiotic. Although intrinsic bile tolerance appear to be strain-dependent, both lactobacilli and bifidobacteria can progressively adapt to the presence of bile salts, and resistant derivatives can be obtained from sensitive wild type strains by subculturing in gradually increasing concentrations of bile (Noriega et al., 2004; Guglielmotti et al., 2007; Burns et al., 2010). On some occasions, bile salt-resistant strains can also be obtained by selection toward

other stress conditions, such as acid pH (Chou and Weimer, 1999); and bile-adapted strains usually display cross-resistances to other stress factors (Margolles et al., 2003). Indeed, this reflects the existence of common mechanisms in bacterial responses to various stresses and suggests that enhancing probiotics bile tolerance could help to develop more robust strains displaying enhanced resistance to other technological or gastrointestinal factors compromising probiotics survival (Sánchez et al., 2012). Bile-adapted strains also provide an interesting model to analyze the molecular mechanisms involved in bacterial tolerance and response to these compounds. Indeed, by using high-throughput techniques on some of these bacterial models some pivotal aspects mediating bile resistance and response in these microorganisms have been identified (Sánchez et al., 2007b; Burns et al., 2010). Overall, bile response is a multifactorial phenomenon, implicating a variety of processes addressed toward detoxification of bile and counteracting the deleterious effect on bacterial structures, as described on the following paragraphs. Active efflux of bile acids/salts (Pfeiler and Klaenhammer, 2009; Bustos et al., 2011; Ruiz et al., 2012a,b), bile salt hydrolysis (Kumar et al., 2006; Lambert et al., 2008), and changes in the architecture/composition of cell membrane and cell wall (Gómez-Zavaglia et al., 2002; Taranto et al., 2003; Ruiz et al., 2007) appear to be the most prevalent bile-specific mechanisms mediating resistance in both genera. In addition, general stress response, protection against oxidative damages, as well as global glycolytic reorganizations are other common consequences of bile exposure, that might be employed to counteract some of the cellular damage caused by these compounds (**Figure 1**; **Table 1**; Hamon et al., 2011; Ruiz et al., 2011; Alcantara and Zuñiga, 2012).

ROLE OF BILE-EFFLUX SYSTEMS

The active extrusion of the bile acids and salts that accumulate in the cytoplasm through efflux pumps is a common bacterial mechanism to counteract bile toxicity (Piddock, 2006). To date, a number of multidrug transporters (MDRs) belonging to the ATP-binding cassette or the major facilitator superfamily, have been described to mediate bile tolerance in lactobacilli and bifidobacterial strains: four transporters in *Lactobacillus acidophilus* NCFM, LBA0552, LBA1429, LBA1446, and LBA1679 (Pfeiler and Klaenhammer, 2009); one in *Lactobacillus reuteri* ATCC 55730, *lr1584* (Whitehead et al., 2008); two in *Bifidobacterium longum*, *ctr* and *BL0920* (Price et al., 2006; Gueimonde et al., 2009), and one in *Bifidobacterium breve*, *Bbr_0838* (Ruiz et al., 2012a,b). Indeed, deletion of any one of LBA0552, LBA1429, LBA1446, and LBA1679 transporters in the strain *L. acidophilus* NCFM rendered the mutant strains more sensitive to bile and certain antibiotics (Pfeiler and Klaenhammer, 2009); and mutation of *lr1584* in *L. reuteri* reduced the strain capability to grow in the presence of bile and completely abolished its capacity to acquire bile-tolerant phenotypes (Whitehead et al., 2008). In bifidobacteria, only *Bbr_0838* has been inactivated, through

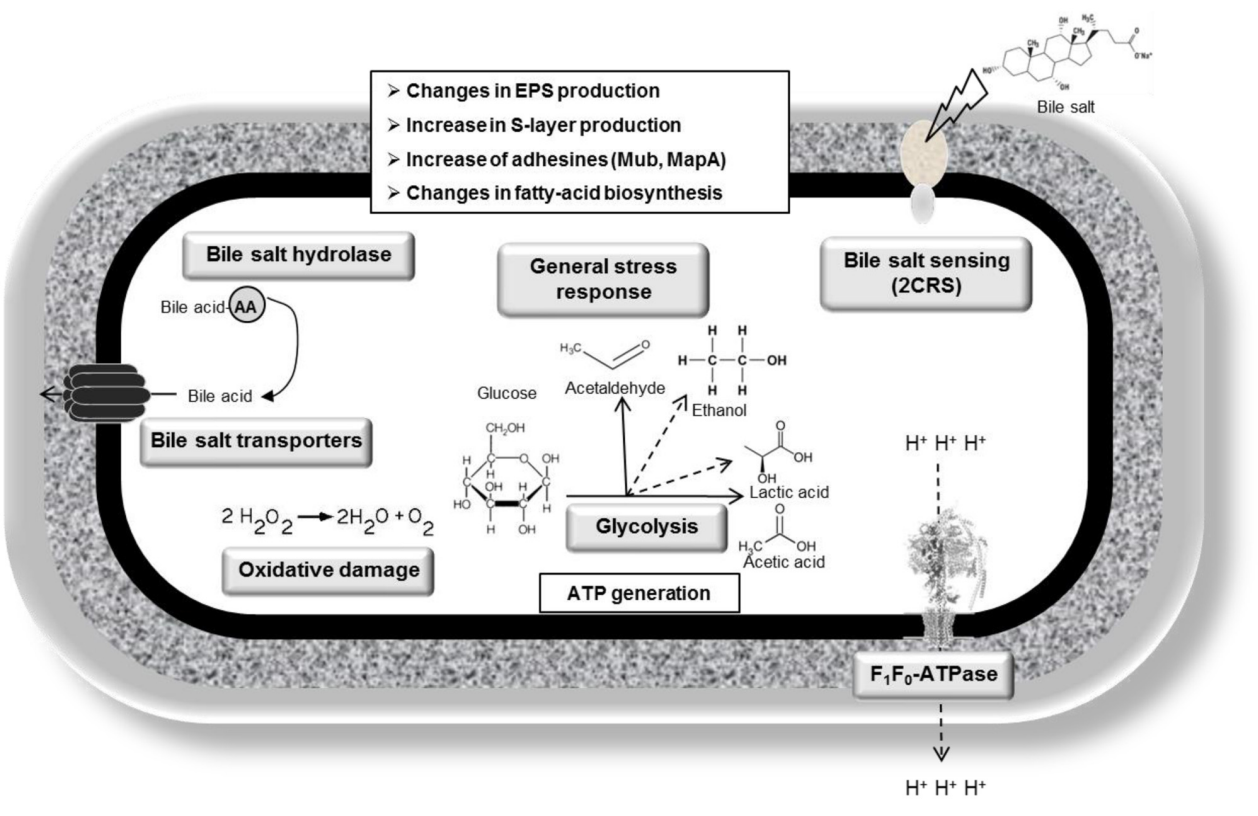

FIGURE 1 | Bile response mechanisms identified and characterized in lactobacilli.

Table 1 | Strategies and molecular elements involved in bifidobacterial bile response and adaptation. Bile induced (+); bile repressed (−).

Strategy	Molecular mechanisms and players	Species	Reference
Bile salt deconjugation (unclear)			
	bsh	*B. animalis*	Kim and Lee (2008)
Bile efflux			
	Bbr_0838 (+)	*B. breve*	Ruiz et al. (2012a,b)
	ctr (constitutive)	*B. longum*	Price et al. (2006)
	betA (+)	*B. longum*	Gueimonde et al. (2009)
Counteracting H+ accumulation			
	F_0F_1-ATPase (+)	*B. animalis*	Sánchez et al. (2006)
General stress response			
	HtrA, DnaK, GroEL	*B. animalis and B. longum*	Sánchez et al. (2008)
Counteracting redox state	Methionine synthase, peroxidase	*B. longum*	Sánchez et al. (2007a)
Surface properties			
Surface proteome	DnaK (+) – (colonization factor?)	*B. animalis*	Candela et al. (2010)
	Enolase (+) (colonization factor?)	*B. longum*	Ruiz et al. (2009)
	OppA (+)	*B. longum*	Ruiz et al. (2009)
EPS	*p-gtf* (+)	*B. animalis*	Ruas-Madiedo et al. (2009)
	EPS production related to bile tolerance	*B. breve*	Fanning et al. (2012)
	EPS production related to bile tolerance	*B. breve, B. bifidum and B. pseudocatenulatum*	Alp and Aslim (2010)
Fatty acids	Bile response and adaptation related to changes in fatty acids composition	*B. animalis*	Ruiz et al. (2007)
	Bile response related to changes in fatty acids composition	*B. bifidum and B. pseudolongum*	Gómez-Zavaglia et al. (2002)
Carbon metabolism			
Fluctuation fermentable carbon sources	Glycosidases	*B. animalis*	Ruas-Madiedo et al. (2005)/ Noriega et al. (2004)
Increase ATP production	Glycolitic enzymes (+)	*B. longum*	Sánchez et al. (2005)
	F6PPK/GA3PDH (+)	*B. animalis*	Sánchez et al. (2007b)
Others			
	ABC-type and MDR transporters	*B. breve*	Ruiz et al. (2012b)

insertional mutation, a change that reduced the strain's capability to grow in the presence of cholic acid (Ruiz et al., 2012a). Previous results on the BL0920 transporter from *B. longum*, which shares a high degree of homology with *Bbr_0838*, also suggest a role in bile protection (Gueimonde et al., 2009; Ruiz et al., 2012b).

The active extrusion of labeled bile has been demonstrated for the bifidobacterial transporters BL0920 and ctr (Price et al., 2006; Gueimonde et al., 2009). In *Lactobacillus johnsonii*, a functional taurocholic and cholic acid antiporter belonging to the major facilitator superfamily, CbsT2, has also been described although its contribution to bile tolerance has not been demonstrated by means of functional genetics (Elkins and Savage, 2003). Furthermore, *L. reuteri* efflux of both free and conjugated bile acids has been demonstrated and ATP was found to be the main force driving the extrusion activity (Bustos et al., 2011).

Remarkably, all transporters identified to date in lactobacilli and bifidobacteria mediating bile-tolerance and/or extrusion activity, exhibit some degree of bile-inducibility (Gueimonde et al., 2009; Koskenniemi et al., 2011; Ruiz et al., 2012b). The bifidobacterial genes *BL0920* and *Bbr_0838*, exhibited the highest levels of transcriptional induction following bile exposure (Gueimonde et al., 2009; Ruiz et al., 2012b) and appear to present homologs in bifidobacterial strains of intestinal origin (Gueimonde et al., 2009). Preliminary characterization of their promoter regions identified putative regulatory elements; however, specific transcriptional regulators have yet to be identified. This will significantly contribute to our understanding on the acquisition and evolution of traits conferring a selective advantage within the intestinal environment.

BILE-SALT HYDROLASES

Among the different mechanisms deployed by bacteria to counteract the harmful effect of bile, the activity of bile-salt hydrolases (BSHs) has been proposed to confer protection through bile

salt deconjugation. BSHs belong to the cholloglycine hydrolase family of enzymes, and have been proposed to have evolved as an adaptation to bile-containing environments (Begley et al., 2005; Jones et al., 2008). BSH catalyzes a reaction in which glycine and taurine are de-conjugated from bile salts, and the corresponding unconjugated acids can be further metabolized by other gut bacteria (De Boever et al., 2000). In *Lactobacillus amylovorus* and *Lactobacillus plantarum*, a comparison between wild type and mutated BSH established a link between BSH activity and bile tolerance (Begley et al., 2005). Such comparisons have not been performed on bifidobacterial BSH, however compiling evidence suggest a role of the enzyme in bifidobacterial bile resistance. For instance, BSH appear over-represented in a bile-adapted *Bifidobacterium animalis* strain that also displays higher hydrolyzing activity than its wild type counterpart (Noriega et al., 2006; Sánchez et al., 2007b). Nevertheless, the mechanism by which BSH may confer bile protection is not fully understood since unconjugated forms are more hydrophobic and toxic as they can freely enter the cells, so they need to be actively pumped outside. However, they are weaker acids than their conjugated counterparts, thus recapturing the co-transported proton may counteract the drop in pH that take place in bile environments (Begley et al., 2005). Remarkably, BSH homologs are only present in bile containing environments, reflecting its importance to enhance bacterial competitiveness within the intestine (Jones et al., 2008). However, there is no agreement on its significance for *in vivo* persistence of lactobacilli and bifidobacteria, although this role has been unequivocally proven in other microorganisms like *Listeria* spp. (Kumar et al., 2012).

Bile-salt hydrolase is an inducible activity in *Lactobacillus*, and expression of *bsh* gene in *L. plantarum* was increased sixfold after exposure to 2% bile (Duary et al., 2012). *In vitro* experiments suggested the activity is constitutively expressed in bifidobacteria (Sánchez et al., 2005), although *in vivo* assays revealed intracellular accumulation of this enzyme in *B. longum*, in the gut of rabbits. This pointed to intestinal factors, other than bile, triggering its expression (Yuan et al., 2008) and supports the significance of this activity within the intestinal environment. Lactobacilli and bifidobacteria can harbor several functional copies of BSH genes within their genomes, all of them participating in bile salt deconjugation, with a substrate preference (Ren et al., 2011). Remarkably, BSH specificity seems to rely on the specific amino acid and hence, BSH has also been proposed to confer a nutritional advantage on producing bacteria, through capturing the amino acid moieties released from its hydrolyzing activity (Begley et al., 2005). Interestingly, BSH and bile salt transporters are sometimes found organized in operons, notably in lactobacilli strains isolated from the human environment and not from dairy products (Elkins et al., 2001; Elkins and Savage, 2003).

Gut microbiota BSH activity has also been related to effects on the host. Increases in the BSH levels have been linked to a higher cholesterol-removing capacity, which may be considered beneficial for the human host (Dong et al., 2012). However, unconjugated bile acids are not as well re-absorbed and can be further transformed into secondary bile acids whose accumulation in the colon has been speculated to cause certain tissue damage (Li and Chiang, 2012). Overall, evidence suggests that BSH enzymes play a significant role for gut bacteria, presumably contributing to bile tolerance, although the mechanisms are not completely understood. Similarly, the impact of this gut-microbiota encoded activity on the host needs further investigation.

EFFECTS OF BILE SALTS ON THE BACTERIAL ENVELOPES AND FATTY ACID METABOLISM

Due to its lipophilic character, bacterial membranes represent one of the main targets of bile that disrupts the structure of bacterial envelopes, affecting both cell and colony morphology (Suskovic et al., 2000; Margolles et al., 2003; Kurdi et al., 2006). This effect has been evaluated and used as a bile salt-resistance marker in certain *Lactobacillus* strains, since rough colonies are more sensitive than smooth colonies, probably in connection to changes in envelope architecture (Suskovic et al., 2000). Furthermore, changes in the lipid composition of bacterial membranes have been described following bile exposure in bifidobacteria and lactobacilli (Gómez-Zavaglia et al., 2002; Kociubinski et al., 2002; Taranto et al., 2003; Ruiz et al., 2007). Remarkably, in *B. animalis* IPLA4549 bile has been suggested to promote changes in the composition of the membrane lipids through changes in the production of proteins involved in fatty acid metabolism (Sánchez et al., 2007b). These observations correlate to transcriptomic data of bile-exposed *B. animalis* BB12, and are consistent with studies that demonstrate bile-induced changes in bifidobacterial membrane composition and surface properties (Gómez-Zavaglia et al., 2002; Kociubinski et al., 2002; Savijoki et al., 2005; Ruiz et al., 2007). Similarly, fatty acid changes described in lactobacilli following bile exposure (Taranto et al., 2003) are in agreement with variations in enzymes involved in fatty acid metabolism, as revealed through proteomic and transcriptomic approaches in *Lactobacillus rhamnosus* GG (Koskenniemi et al., 2011). However, these changes appear to be strain-dependent and therefore it is difficult to interpret how they contribute to the defense against bile toxicity. They have been proposed to result in alterations in the physicochemical properties of the membranes and cell wall functionalities which might contribute to reduce bile diffusion (Gómez-Zavaglia et al., 2002; Taranto et al., 2006; Ruiz et al., 2007). For instance, bile exposure in bifidobacteria was associated to increased hydrophobicity and reduced z-potential, due to the bile moieties accumulated within the membranes (Kociubinski et al., 2002). In *B. animalis* IPLA4549 bile adaptation also resulted in a strain with a higher proportion of saturated fatty acids, and displaying reduced membrane fluidity (Ruiz et al., 2007).

Other genes coding for surface-associated proteins, such as mucus-binding protein (*mub*), or mucus adhesion promoting protein (*mapA*) in *L. plantarum* isolates, appeared over-represented when the growing media was supplemented with a mix of mucin (0.05%) and bile (1%; Duary et al., 2012). Similarly, in a *B. longum* strain, surface associated enolase and DnaK, which are able to capture human plasminogen, appeared up-regulated by bile, thus suggesting that bile modulates molecular traits exerting a role in intestinal colonization (Candela et al., 2009, 2010; Ruiz et al., 2009). However, these results could not always be correlated with increased adhesion to intestinal cell lines or mucin. For

instance, a bile salt-adapted strain of *Lactobacillus delbrueckii* subsp. *lactis* showed reductions in cell hydrophobicity, auto-aggregation, and adhesion to human cell lines, despite improved resistance to physiological bile-salt concentrations (Burns et al., 2011a). On the contrary, bile adaptation in *B. animalis*, *B. longum*, and *Bifidobacterium bifidum* strains was correlated with increased adhesion to intestinal mucus *in vitro* and, although the presence of physiological concentrations of bile reduced the adhesion in all cases, the adapted strains still displayed higher binding capacity than their original counterparts (Gueimonde et al., 2007). However, the effect of a simulated gastric transit on pairs of *B. animalis* and *B. longum* strains, including the wild type and their bile-adapted counterparts, showed no improved adherence of bile-adapted strains to intestinal cell lines *in vitro* (de los Reyes-Gavilán et al., 2011). Therefore, further research is needed to confirm whether *in vitro* bile adaptation improves *in vivo* performance.

Production of external exopolysacharide (EPS) layers is an extended trait among intestinal bacteria (Ruas-Madiedo et al., 2007). These exocellular polymers cause a deep impact on bacterial surface properties and act as a protective coat against environmental conditions (Alp and Aslim, 2010; Leivers et al., 2011; Fanning et al., 2012). In accordance with this, bile has been demonstrated to induce exopolysaccharide production in *B. animalis* IPLA4549, probably as a mechanism of bile protection (Ruas-Madiedo et al., 2009). In fact, *in vitro* and *in vivo* models revealed a correlation between EPS production and bile tolerance in other bifidobacteria. For instance, in *B. breve* UCC2003, the EPS coat was essential for bile survival and *in vivo* colonization of the mice gut (Fanning et al., 2012). Nevertheless, the effect of bile on EPS production in lactobacilli is not as clear. Whereas transcriptomic and proteomic data in *L. rhamnosus* GG point to a reduced production of enzymes involved in EPS biosynthesis in bile-containing environments (Koskenniemi et al., 2011) in *L. delbrueckii* no variations were found following bile exposure, although acquisition of stable bile-resistance was correlated to a significant overproduction of enzymes involved in EPS biosynthesis (Burns et al., 2010). It still remains to be determined whether bile exposure affects the composition and properties of the EPS layers. Finally, other cell-wall structures may be affected in response to bile, as in the case of *L. acidophilus*, which increases S-layer protein production at genetic level when cultured in the presence of 0.05% bile (Khaleghi et al., 2010).

Therefore, *in vitro* analyses show that bile deeply affects surface properties of lactobacilli and bifidobacteria, due to changes on cell wall architecture, lipid composition, presence and characteristics of external coats. Some of these changes have been determined to occur at transcriptional level and may affect bacterial capability to interact with the intestinal epithelia. It still remains to be determined whether bile-acquired tolerance and bile regulation of putative colonization factors translate into better *in vivo* probiotic behavior.

GENERAL STRESS RESPONSE

It is known that, in addition to their action as detergents, bile salts impose oxidative stress on bacteria, due to the production of reactive oxygen/nitrogen species (Sokol et al., 1993; Bernstein et al., 1999; Begley et al., 2005). In addition, bile salts deconjugation releases protons, thus causing an intracellular acidification (Begley et al., 2005). Accordingly, some of the pathways activated in bacteria following a bile challenge are those related with general, acid and oxidative stress responses, as revealed by microarray experiments in *L. reuteri, L. rhamnosus, L. plantarum, L. johnsonii,* and *B. breve* (Bron et al., 2004, 2006; Whitehead et al., 2008; Koskenniemi et al., 2011; Ruiz et al., 2012b; Lee et al., 2013), but also by various-omic approaches in other enterobacteria such as *Enterococcus faecalis* (Rincé et al., 2003) or *Salmonella enterica* (Hernández et al., 2012), among others. The aim of this response is to counteract the negative effects of bile at the level of cell wall disorganization, oxidative stress and DNA damage/protein denaturation and intracellular acidification. In fact, bile exposed bacteria overexpress a range of proteins to counteract these effects. Damage to proteins is counteracted through a chaperone/protease mediated response which promotes a quick recycling of damaged proteins and a proper folding of nascent proteins. In bifidobacteria, overproduction of a battery of proteases and chaperones upon either bile response or adaptation has been shown (Sánchez et al., 2005, 2007b, 2008; Savijoki et al., 2005). The range of bile-induced chaperones/proteases was broader in *B. animalis* than in *B. longum*, with three main chaperones common to both species, HtrA, GroEL, and DnaK, the latter also having been implicated in ox-gall adaptation in *Bifidobacterium adolescentis* NCC251 (Schmidt and Zink, 2000). Some chaperones, ClpP, Dps, GroEL, Hsp1, and Hsp3, were also found to be up-regulated in *L. plantarum* (Hamon et al., 2011). In agreement with this, mutations in the Clp chaperone in *L. reuteri* were also associated with reduced survival in the presence of bile (Whitehead et al., 2008). In the case of *L. acidophilus*, a decrease in H_2O_2 formation was also observed after treatment with 0.1% bile and, although the molecular mechanisms responsible of this effect have not been discerned yet, this suggests that activities aimed at reducing production of oxidant molecules could enhance bile tolerance (Khaleghi et al., 2010). In fact, a DPS protein (DPS: DNA-binding protein from starved cells) and a thioredoxin-dependent thiol peroxidase, both involved in SOS response, are overproduced in a bile-exposed *B. animalis* subsp. *lactis* strain (Sánchez et al., 2007b). Also, co-expression of catalase gene *katA* from *Lactobacillus sakei* and the bile salt hydrolase gene *bsh1* from *L. plantarum* in *Lactobacillus casei* HX01, resulted in higher resistance to both oxidative and bile salt stress (Wang et al., 2011). Furthermore, the F_0F_1ATPase responsible of ATP generation while pumping protons outside the cells, has been described as the molecular link connecting both acid and bile stress responses in *B. animalis* (Sánchez et al., 2006). In fact, F_0F_1ATPase has been found to be up-regulated under bile environments in a variety of bacteria (Hamon et al., 2011; Koskenniemi et al., 2011) and seems to play a crucial role in maintaining the intracellular pH under bile environments.

Two-component regulatory systems (2CRS) have been implicated in sensing bile salt presence in *L. acidophilus* (Pfeiler et al., 2007). An operon encoding a 2CRS, a transporter, an oxidoreductase and four hypothetical proteins was shown to be over-expressed as a response to bile in *L. acidophilus* by transcriptomics. Interestingly, mutations in the genes coding for the 2CRS, in the transporter and in one of the hypothetical proteins resulted in

lower bile salt resistance, while mutations in the oxidoreductase and in another hypothetical protein induced an increase in bile salt tolerance (Pfeiler et al., 2007). The involvement of 2CRS in sensing bile salts has also been described in enterobacteria (Kus et al., 2011). In bifidobacteria, no bile-sensing systems or specifically bile controlled transcriptional regulators have been identified yet.

CHANGES IN CENTRAL METABOLIC PATHWAYS. A FOCUS ON SUGAR METABOLISM

Reorganizations in the global metabolism, notably at the glycolytic level and aimed to enhance energy production seem to be crucial in the response of bifidobacteria and lactobacilli to bile. By increasing energy production, active responses against the detrimental action of bile at different levels, such as bile efflux, fatty acid biosynthesis and cell-wall architecture can be accomplished. However, particular metabolic shifts seem to be strain-dependent. Key enzymes of central metabolism such as phosphofructokinase, phosphoglycerate mutase, or elongation factor Tu were significantly over-expressed in response to bile salts in lactobacilli (Wu et al., 2010). Changes in the glycolytic metabolism, analyzed by measuring end-products, also pointed to a deep metabolic reorganization in lactobacilli as response to bile, suggesting an activation of central glycolysis (Lee et al., 2008; Burns et al., 2010). *B. animalis* and *B. longum* also demonstrated metabolic shifts in carbohydrate metabolism under bile environments, although the particular response appears to be strain-dependent. While *B. longum* accumulates most of the enzymes of the glycolytic pathway, suggesting an increase of glucose consumption in bile environments, *B. animalis* subsp. *lactis* displayed an accumulation of enzymes involved in the formation of fructose-6-phosphate, fructose-6-phosphate-phosphoketolase, and glyceraldehido-3-phosphate dehydrogenase, these being the only overproduced enzymes of the bifid shunt (Sánchez et al., 2005, 2007b). Physiological analysis confirmed an increased rate of glucose consumption in *B. longum* bile exposed cells but not in *B. animalis.* Therefore an increase in ATP production following bile challenge seems to occur through different routes: while *B. longum* increases ATP production through glycolysis, *B. animalis* increases the phosphorylation at substrate level (Sánchez et al., 2008).

Acquisition of bile tolerance was also associated to metabolic shifts in both bifidobacteria and lactobacilli strains. For instance, a bile-resistant *B. animalis* derivative, exhibited a maltose over glucose preference as compared to the parental strain, what might represent a selective advantage within the distal colon, where glucose is not available (Ruas-Madiedo et al., 2005). In addition, a proteomic comparison of bile response in both wild type and derivative strains, suggests that in the bile-adapted strain, the bifid shunt is displaced toward other metabolic pathways, i.e., oxalic degradation, that would theoretically increase ATP production (Sánchez et al., 2007b). Indeed, under bile environments, a bile-adapted *B. animalis* maintains a higher ATP concentration than its original counterpart (Sánchez et al., 2006). In *Lactobacillus*, bile adaptation resulted in higher glucose consumption and lactic acid formation, as compared to wild type strains (Burns et al., 2010). Interestingly, a bile-salt adapted strain of *L. delbrueckii* subsp. *lactis* decreased the production of ethanol through the glycolytic pathway, with a concomitant increase of aroma-related compounds, such as acetaldehyde, when grown in milk and with respect to the parental strain (Burns et al., 2011b). Therefore, care should be taken in the sense that bile-salt adaptation cannot only affect probiotic traits such adhesion (Burns et al., 2011a), but also important properties in food technology, such as variations in production of metabolic end products or resistance to bacteriophages (Guglielmotti et al., 2007; Burns et al., 2011b).

CONCLUSION

Bile plays an important role in the physiology of intestinal bacteria, thus conditioning their functionality. This is particularly important for probiotic bacteria, since their beneficial effects must be generated in the presence of this biological fluid. In fact, we know that the activities of intestinal lactobacilli and bifidobacteria are deeply influenced by the presence of bile salts, and even some of them, such as cholesterol assimilation, have been directly correlated with bile salt metabolism in these bacteria. The understanding of the mechanisms by which probiotic bacteria are able to survive the stress caused by bile salts has remained elusive, but current -omics techniques have unraveled the protein and gene networks involved in this process, and delineated specific responses directed to cope with bile stress. It is remarkable that the existence of common mechanisms to cope with bile stress in probiotic bacteria belonging to phylogenetically different groups as is the case for *Bifidobacterium* and *Lactobacillus*, reflecting the existence of convergent evolutionary forces that have shaped probiotic tools to compete within the intestinal environment. Activation of molecular machinery to counteract oxidative and acid stresses are common responses to bile stress, as well as utilization of bile efflux systems and bile modification through bile salt hydrolases. Application of -omics methodologies to analyze strains performance *in vivo*, will undoubtedly shed valuable information to identify key players in which we can act in order to improve the survival of probiotics along the gastrointestinal tract.

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Of energy and survival incognito: a relationship between viable but non-culturable cells formation and inorganic polyphosphate and formate metabolism in *Campylobacter jejuni*

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Campylobacter jejuni is a Gram-negative food-borne bacterium that can cause mild to serious diseases in humans. A variety of stress conditions including exposure to formic acid, a weak organic acid, can cause *C. jejuni* to form viable but non-culturable cells (VBNC), which was proposed as a potential survival mechanism. The inability to detect *C. jejuni* VBNC using standard culturing techniques may increase the risk of exposure to foods contaminated with this pathogen. However, little is known about the cellular mechanisms and triggers governing VBNC formation. Here, we discuss novel mechanisms that potentially affect VBNC formation in *C. jejuni* and emphasize the impact of formic acid on this process. Specifically, we highlight findings that show that impairing inorganic polyphosphate (poly-P) metabolism reduces the ability of *C. jejuni* to form VBNC in a medium containing formic acid. We also discuss the potential effect of poly-P and formate metabolism on energy homeostasis and cognate VBNC formation. The relationship between poly-P metabolism and VBNC formation under acid stress has only recently been identified and may represent a breakthrough in understanding this phenomenon and its impact on food safety.

Keywords: viable but non-culturable cells, *Campylobacter jejuni*, inorganic polyphosphate, polyphosphate kinase, formate metabolism, formate dehydrogenase, acid stress, energy

ENTER VBNC: A BRIEF HISTORY, SIGNIFICANCE, AND CONTROVERSY

Researchers in 1982 observed that two bacterial species, *Escherichia coli* and *Vibrio cholerae*, could not be retrieved from saltwater microcosms using a medium that previously sustained their growth (Xu et al., 1982). Although these bacterial species lost culturability in response to stress, they still maintained detectable metabolic activity, which suggested that these cells were still viable (Xu et al., 1982). Based on these observations, researchers suggested that stressed bacterial cells might exist in a viable but non-culturable (VBNC) state (Colwell et al., 1985; Oliver, 2010). Since the initial discovery, there have been hundreds of publications documenting VBNC formation in a variety of bacterial species, including important pathogens such as *Helicobacter pylori*, *V. cholerae*, *Campylobacter jejuni*, and others (Oliver, 2005). Further, it was shown that VBNC can be induced under different stresses, including exposure to chlorine, acids, oxygen, and pasteurization as well as those associated with fluctuations in the environment's salinity, pH, and temperature (Chaveerach et al., 2003; Oliver, 2005). Today, VBNC are broadly defined as cells that enter a non-culturable state in response to stress, while maintaining a detectable but reduced metabolism (e.g., decrease in respiration, nutrient transportation, and synthesis of macromolecules), relatively high ATP levels, and aspects of cellular integrity such as intact chromosome content and cell membrane (Grégori et al., 2001; Oliver, 2005, 2010). Additional VBNC characteristics also occasionally include changes to cell morphology such as "rounding up" and reduction in the size of the cells, which is thought to maximize the surface area available for nutrient uptake while minimizing cell mass (Colwell, 2000; Oliver, 2005). It is important to note that the VBNC state may differ from other survival mechanisms. For example, in *Enterococcus faecalis*, the proteomic profiles of starved cells were observed to be different from those of the VBNC (Heim et al., 2002), which potentially indicated that the latter was triggered only in response to certain stresses.

With increasing knowledge about VBNC, their significance as a potential risk for public health became evident. A major concern is the inability to detect pathogens in the VBNC state using standard culture-based techniques. This is significant, because VBNC can potentially retain virulence and can be resuscitated back to "normal"/culturable physiological state under favorable conditions, including those available within hosts (Oliver, 2005, 2010). Subsequently, this may increase the potential for undetectable contamination and the spread of infectious agents to susceptible hosts. Although this is a contentious issue with arguments and research either supporting or disproving the ability of VBNC to cause disease in hosts, it is important to note that the possibility for infection should not be merely disregarded (Oliver, 2005, 2010). The lack

of complete knowledge in regards to VBNC's virulence, factors influencing their resuscitation, and cognate public health risks and ramifications might at the very least provide supportive impetus for prudence when considering risks associated with VBNC. In fact, under certain scenarios, potential risks may weigh heavily and can pose a severe threat to public welfare. For example, VBNC contamination of food products (Rowan, 2004; Dinu and Bach, 2011) and medical equipment (Zandri et al., 2012) can go undetected, impacting consumers, jeopardizing the safety of food, and threatening the lives of susceptible patients.

Of particular interest for food safety is *C. jejuni*, a food-borne pathogen that can form VBNC under stress (Rollins and Colwell, 1986; Jackson et al., 2009). *C. jejuni* is a Gram-negative bacterium that can cause disease in humans, including gastroenteritis and occasionally debilitating and life-threatening neuropathies (Vandamme and De Ley, 1991; On, 1996). The control of *C. jejuni* in poultry and other food animals and products (e.g., beef, turkey, and milk) has proved to be challenging, due in part to the atypical pathobiology of this bacterium, which lacks many of the classical stress response factors associated with other enteric pathogens (Parkhill et al., 2000). This singularity of *C. jejuni* necessitates a closer consideration of all its possible survival strategies, including VBNC formation; in order to enhance on-going efforts to reduce this pathogen in foods. This viewpoint is supported by research showing that *C. jejuni* VBNC can adhere to the skin of chicken carcasses (Jang et al., 2007), while a recent study reported that *C. jejuni* VBNC can still express a protein (CadF) that facilitates its attachment to host cells (Patrone et al., 2013). Further, it was also shown that *C. jejuni* VBNC can colonize suckling mice (Jones et al., 1991). Therefore, in this minireview, we will briefly discuss some of the molecular factors involved in VBNC formation in bacteria and focus in more detail on *C. jejuni*, highlighting recent research that associates specific metabolic pathways with VBNC formation in this important pathogen.

UNTHREADING THE MYSTERY: GENETIC FACTORS INVOLVED IN VBNC FORMATION AND VIRULENCE

To date, many of the cellular triggers and genetic factors involved in VBNC formation are not well understood. However, increasing research into this phenomenon has revealed glimpses of potential factors that are likely involved in the persistence and expression of virulence in VBNC. Notably, it has been reported that gene expression in VBNC can continue for extended periods of time; for instance, the cytotoxin–hemolysin (*vvhA*) transcripts were detected in VBNC of *V. vulnificus* for up to 4.5 months (Saux et al., 2002). Although it is not clear if these expressed genes are directly involved in VBNC formation, the latter example highlights the possibility for maintaining virulence in the VBNC state. Further, many studies reported the expression of virulence-associated genes in VBNC of other pathogens. For example, in a recent study the expression of *cadF*, a gene that encodes an outer membrane protein that facilitates binding to fibronectin in host cells, was detected at high levels in *C. jejuni* VBNC for 3 weeks (Patrone et al., 2013). In parallel to these observations, the authors also reported that *C. jejuni* VBNC were capable of adhering to intestinal cells *in vitro*, but at levels that were lower than that of the culturable strain (Patrone et al., 2013). In another study, virulence-associated genes, including those encoding flagellin proteins, the cytolethal distending toxin, and a *Campylobacter* invasion antigen that are involved in invading- / interacting with the host's intestinal cells were found to maintain a low level of expression in *C. jejuni* VBNC (Chaisowwong et al., 2012). Similarly, the mRNA of the Shiga toxin encoding gene (*stx1*) was detected in VBNC of *E. coli* O157:H7 (Yaron and Matthews, 2002), which is a notable finding because these toxins are associated with hemorrhagic colitis, hemolytic uremic syndrome, thrombocytopenia, hemolytic anemia, and renal failure (Karmali, 1989). Coccoid-shaped cells of *V. cholerae* entering a VBNC state were found to express the toxin co-regulated pilus (TCP), a virulence factor that is important for colonization of the small intestine in humans, and were able to colonize infant mice (Krebs and Taylor, 2011). The authors also noted that in a previous study TCP like appendages could be seen in micrographs of 1 year old *V. cholerae* VBNC (Chaiyanan et al., 2007). Another investigation detected viable-non-culturable and coccoid-shaped cells of *H. pylori* in biopsies collected from 12 dyspeptic patients, and these cells expressed *luxS*, a gene associated with quorum sensing and bacterial virulence (Cellini et al., 2008). Collectively, the aforementioned studies and other published research (**Table 1**) support the ability of VBNC to maintain some aspects of virulence and/or regain them after resuscitation.

It was shown that two regulatory genes (*algU* and *gacA*) that code for the alternative sigma factor (σ^E) and a response regulator, respectively, may be involved in VBNC formation in *Pseudomonas fluorescens* CHA0, which is used as a biocontrol agent against black root rot (Mascher et al., 2002). Additionally, it was suggested that resuscitation-promoting factor (Rpf)-like proteins might be involved in the reactivation of non-culturable cells of the human pathogen *Mycobacterium tuberculosis* (Shleeva et al., 2002). A delay in VBNC formation in an *S.* Typhimurium LT2 mutant was associated with a 99-bp in-frame deletion in the *clpX* gene, which is known to be involved in forming a protease complex that degrades the general stress sigma factor RpoS (Kusumoto et al., 2013). Subsequently, the authors suggested that this ClpX–RpoS relationship might have affected entry into the VBNC state (Kusumoto et al., 2013). Further, RpoS expression was detected for up to 14 days in VBNC of *V. vulnificus* (Smith and Oliver, 2006), while this stress factor was implicated in the persistence of *E. coli* in a VBNC state (Boaretti et al., 2003). In another study, the inactivation of OxyR, an oxygen stress regulator, and the cognate catalase enzyme impacted VBNC formation in *V. vulnificus* (Kong et al., 2004). Collectively, these are very interesting findings and can potentially shed light on the VBNC state of important pathogens and beneficial bacteria; however, this also raises several important questions. For example, the atypical pathogen, *C. jejuni*, has substantially documented VBNC state but lacks RpoS, OxyR, and a σ^E response (van Vliet et al., 1999; Parkhill et al., 2000), while investigations of a potential *C. jejuni* resuscitation factor (Cj0645) in strain NCTC11168 showed that the target was not an Rpf ortholog (Morgan, 2010). These observations suggest that the aforementioned genes may not necessarily be a factor in all VBNC-forming pathogens, which raises the following question: could there be a ubiquitously distributed cellular mechanism that might affect VBNC formation across many species? This question can perhaps be partially addressed by recent findings (detailed below) that

Table 1 | Example of studies that investigated the factors that trigger VBNC formation in *C. jejuni* and possible approaches to resuscitate these cells.

Reference	VBNC inducing factor(s)	Resuscitation	Expression of virulence genes	Other
Patrone et al. (2013)	Incubation in freshwater microcosms at 4°C	NA	*cadF* (mediates binding to fibronectin)	Adherence to human intestinal cells (Caco-2) *in vitro*
Chaisowwong et al. (2012)	Cold stress (4°C) in a nutrient rich medium (Bolton broth)	Co-incubation with Caco-2 in some experiments	Flagellar genes (*flaA*, *flaB*), *cadF*, *Campylobacter* invasion antigen gene (*ciaB*), cytolethal distending toxin genes (*cdtA*, *cdtB*, and *cdtC*)	Invasion of Caco-2 cells
Gangaiah et al. (2010)	Formic acid in Mueller-Hinton broth at 42°C	NA	NA	NA
Klancnik et al. (2009)	Short-term starvation (5 h incubation in a low nutrient medium)	NA[1]	NA	*In vivo* systemic campylobacteriosis in mice[1]. Adhesion, invasion, and survival in Caco-2 for up to 4 days[1]. Heat-stress resistance (55°C for 3 min)[1]
Gangaiah et al. (2009)	Formic acid in Mueller-Hinton broth at 42°C	NA	NA	NA
Guillou et al. (2008)	Storage in bottled water at 4°C in the dark	Inoculation into chicken embryonated eggs	NA	NA
Jang et al. (2007)	Aerobic conditions at 4, 25, and 37°C	NA	NA	Found after rinsing on artificially inoculated crevices and feather follicles of chicken skin[2]
Tangwatcharin et al. (2006)	Cold (4°C in *Brucella* broth) and heat-stress (60°C in brain heart infusion broth)	NA	NA	Some loss in the outer membranes of aging cell suspensions
Baffone et al. (2006)	Artificial sea water at 4°C	*In vivo* passage in the mouse intestine (dependent on the titer of respiring bacteria in the VBNC state; $>10^4$ cell/ml)	NA	Colonization of mice
Ziprin and Harvey (2004)	Sterile water at room temperature	Failure to resuscitate in day-of-hatch leghorn and broiler chicks with experimentally introduced normal gut microflora	NA	No colonization of the chicken ceca 7 days post-VBNC inoculation

(Continued)

Table 1 | Continued

Reference	VBNC inducing factor(s)	Resuscitation	Expression of virulence genes	Other
Ziprin et al. (2003)	Sterile water at room temperature	Failure to resuscitate in day-of-hatch leghorn chickens 1 and 2 weeks after inoculation	NA	No colonization of the chicken ceca
Chaveerach et al. (2003)	Mueller-Hinton broth with formic acid (pH = 4.0)	Inoculation into specific-free-pathogen fertilized chicken eggs	NA	Colonization of embryonated eggs
Thomas et al. (2002)	Simulated aquatic conditions at 10°C	NA	NA	NA
Cappelier et al. (1999)	Starvation in sterilized surface water (pH = 6.0) at 4°C	Inoculation into yolk sacs of embryonated eggs	NA	Ability to adhere to HeLa cells after resuscitation
Tholozan et al. (1999)	Starvation in sterilized surface water (pH = 6.0) at 4°C	NA	NA	Increase in VBNC cell volume, decrease in internal potassium content and the membrane potential. Only AMP was detected after 30 days of incubation
Lazaro et al. (1999)	Suspension in phosphate-buffered saline (pH = 7.3) in the dark at 4 or 20°C	NA	NA	Up to 7 months of viability. Intact chromosomal DNA (after 116 and 61 days at 4 and 20°C, respectively). Bleb-like membrane vesicles around cells at 4°C
van de Giessen et al. (1996)	Suspension in sterilized surface water and potassium phosphate buffer at 4°C	Failure to resuscitate in chickens and mice	NA	No colonization of the ceca and intestines of the chickens and mice
Stern et al. (1994)	Suspension in phosphate-buffered saline (pH = 7.2) at 4°C	Resuscitation in 2 out of 39 one-day old chickens	NA	Colonization of the ceca of some chickens
Medema et al. (1992)	Starvation in filter-sterilized and pasteurized surface water	Failure to resuscitate in 1-day old chickens and the allantoic fluid of embryonated eggs	NA	No colonization of the chicken ceca
Jones et al. (1991)	Sterilized pond water at 4°C	*In vivo* passage in suckling mice (only two strains out of four were retrieved)	NA	NA

Information about virulence properties and gene expression in the VBNC is also highlighted. [1,2] *It was not clear if the authors in these studies used a culture/suspension that only contained VBNC cells.NA, not applicable.*

link VBNC formation in *C. jejuni* to the metabolism of inorganic polyphosphate (poly-P), an ancient molecule that is ubiquitous in bacteria and plays a role in energy storage and production (Kornberg et al., 1999; Rao et al., 2009).

Despite the current gaps in knowledge, the studies highlighted previously present a convincing case for researching the virulence of VBNC and their potential impact on public health. This might be of critical relevance when considering the survival mechanisms of atypical pathogens such as *C. jejuni* and cognate ramifications to public health, including food safety.

OF ENERGY AND VBNC: A RELATIONSHIP BETWEEN *C. jejuni* VBNC FORMATION AND INORGANIC POLYPHOSPHATE METABOLISM

Most of the past work that focused on *C. jejuni* VBNC mainly described the physical, chemical, and environmental triggers that induce this state such as exposure to oxygen, persistence in aquatic microcosms, changes in temperature and pH, and starvation (Jackson et al., 2009; **Table 1**). In addition, there was an emphasis on strategies aiming at resuscitation of *C. jejuni* VBNC *in vitro* or *in vivo* (Bovill and Mackey, 1997; Cappelier et al., 1999; Chaveerach et al., 2003; Baffone et al., 2006; **Table 1**). Like in many VBNC-forming bacteria, the genetic mechanisms that are associated with the VBNC state of *C. jejuni* are largely unknown. However, it was recently shown that VBNC formation in *C. jejuni* might be impacted by proteins involved in the metabolism of inorganic poly-P. Specifically, poly-P is a linear polymer of orthophosphate residues that plays a vital role in *C. jejuni* and other bacteria as a source of ATP for approximately 500 cellular reactions and as a modulator of stress and survival phenotypes (Kornberg et al., 1999; Rao et al., 2009; Kassem and Rajashekara, 2011). Since (1) maintaining cellular respiration and relatively high ATP levels are two major features of the VBNC state, (2) conserving energy is a basic survival strategy under stress, and (3) *C. jejuni*, a bacterium with relatively small genome that lacks typical stress responses, has invested in retaining a network of enzymes associated with poly-P metabolism, a link between the poly-P molecule and *C. jejuni* VBNC appeared to be plausible. The latter mandated a closer look at *C. jejuni* that revealed that this pathogen possessed two major enzymes, namely polyphosphate kinase 1 (PPK1) and polyphosphate kinase 2 (PPK2), which are principally involved in the synthesis/accumulation of poly-P and associated GTP production, respectively (Gangaiah et al., 2009, 2010). The inactivation of these enzymes leads to pleiotropic effects, influencing different survival phenotypes in *C. jejuni* (Gangaiah et al., 2009, 2010). Notably, live/dead cell staining analysis showed that the *C. jejuni* deletion mutants, Δ*ppk1* and Δ*ppk2*, possessed a significantly reduced ability to form VBNC after challenge with formic acid (Gangaiah et al., 2009, 2010). This was confirmed using flow cytometry analysis that revealed a significant change in the cell size and granularity of the Δ*ppk1* mutant as compared to the parental strain (Gangaiah et al., 2009), possibly indicating an increase in dead cells in the acid-stressed mutants (Kusters et al., 1997). While investigating the expression of a number of genes that were believed to contribute to the phenotypes of the Δ*ppk1* mutant, it was found that the phosphate regulon genes (*phosR*, *pstS*, *pstC*, and the periplasmic substrate binding protein-encoding gene, CJJ81176_0750), the multidrug resistance efflux pump gene (*cmeC*), the global post-transcriptional regulator (*csrA*), and the stringent response regulator (*spoT*) were not affected in the acid-stressed mutant cells (Gangaiah et al., 2009). However, *ppk2* was significantly down regulated in the formic acid challenged Δ*ppk1* mutant, but the expression of *ppk2* was not affected in similarly treated wild-type cells, which further implicates the *ppks* in *C. jejuni*'s VBNC formation (Gangaiah et al., 2009). Since the Δ*ppk1* mutant was deficient in the accumulation of poly-P and the *ppk2* down regulation would possibly reduce the associated GTP production (Gangaiah et al., 2009), the aforementioned observations suggest that the poly-P-dependent ATP/GTP pools and ratios in the *ppk* mutants might be deficient as compared to that of the parental strain. This assumption is supported by the lower levels of poly-P-dependent GTP and the higher ATP:GTP ratios that were detected in the Δ*ppk2* cells using thin layer chromatography analysis (Gangaiah et al., 2010). Therefore, it appears that the disruption of the poly-P associated enzymes creates an imbalance in the cellular poly-P-dependent ATP/GTP homeostasis, hence affecting the ability of *C. jejuni* to enter the VBNC state. Notably, it was shown that a putative ATP synthase was down regulated in the VBNC of *Enterococcus faecalis* (Heim et al., 2002), which prompted the speculation that the survival of VBNC under unfavorable conditions required alternative metabolic pathways to maintain energy (Heim et al., 2002). This possibly includes using energy that was stored as poly-P, further suggesting that an intact poly-P metabolism is essential for VBNC formation/persistence.

It was reported that poly-P occurs in starved and morphologically altered *V. parahaemolyticus* (Chen et al., 2009), and this molecule also accumulated in structurally intact coccoid forms of starved *H. pylori* (Nilsson et al., 2002). However, no direct mechanistic association between poly-P and the formation of VBNC has been described previously. Although further studies are needed to formulate a comprehensive mechanistic model of the involvement of poly-P and its enzymes in VBNC formation, the advances highlighted above might direct and facilitate future research into the VBNC state.

THE FORMATE CONNECTION: FORMATE METABOLISM AND VBNC FORMATION IN *C. jejuni*

The findings linking poly-P to VBNC formation in *C. jejuni* will generate many extrapolations and questions. For example, the ATP/GTP pools in the cell are not only affected by poly-P and its enzymes; hence, could there be other factors impacting this pool and also contributing to VBNC formation in *C. jejuni*? Further, the Δ*ppk2* mutant only responded to formic acid with reduced VBNC formation as compared to the parental strain, however, there was no difference in the colony forming units (CFU) counts between the mutant and the wild-type after challenge with other organic acids (acetic acid and propionic acid) and hydrochloric acid (Gangaiah et al., 2010). Could the latter observation suggest some specific relationship between formic acid and VBNC formation in *C. jejuni*? As a matter of fact, the previous two questions are intimately linked, because *C. jejuni* possesses a highly branched respiratory chain that serves in energy production

(Myers and Kelly, 2004), while one of the major energy sources for this bacterium is formate that is metabolized by the periplasmic respiratory protein, formate dehydrogenase (Hoffman and Goodman, 1982; Weerakoon et al., 2009). This is not surprising because formate is a byproduct of fermentation that occurs in the hosts' gut, which is the preferable niche for *C. jejuni* (Weerakoon et al., 2009). It was also reported that the inactivation of the formate dehydrogenase (*fdh*) in this bacterium resulted in round shaped cells similar to those associated with the VBNC (**Figure 1A**); however, the *fdh* mutant did not lose culturability under normal growth conditions (Kassem et al., 2012). Further, when the *fdh* mutant was challenged with formic acid as described earlier, it showed a significant decrease in culturability and viability as compared to the wild-type, indicating a severe inability to form VBNC (unpublished data; **Figure 1B**). Therefore, it can be concluded that formate dehydrogenase and formate metabolism are associated with VBNC formation in *C. jejuni*, likely via their role in energy production. Along these lines, a question worthy of investigation is whether the energy produced by formate metabolism may be linked to poly-P accumulation/degradation? Regardless, both the formate and poly-P metabolism are associated with energy and VBNC production in *C. jejuni*, further confirming the role of energy conservation in VBNC formation and subsequent survival.

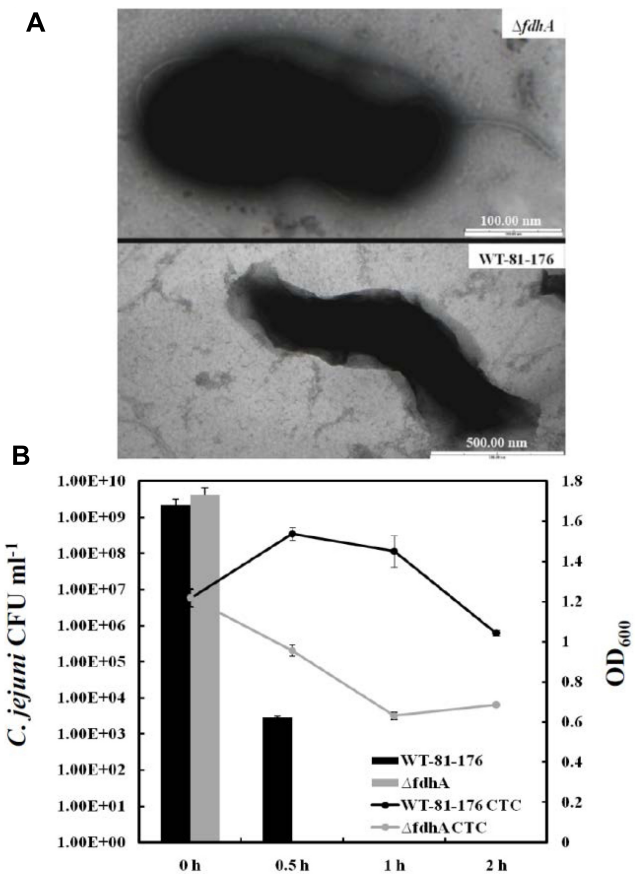

FIGURE 1 | (A) Transmission electron microscopy images of the *C. jejuni* 81-176 wild-type strain and its formate dehydrogenase mutant (Δ*fdhA*) that is defective in the metabolism of formate. The Δ*fdhA* cells exhibited a "rounding up"/spherical morphology, which was also observed in our previous work analyzing the Δ*fdhA* of the *C. jejuni* NCTC-11168 wild-type strain (Kassem et al., 2012). **(B)** CFU enumerations and viability curves of the *C. jejuni* 81-176 wild-type and the Δ*fdhA* after exposure to formic acid (pH = 4.0; Kassem and Rajashekara, unpublished). The CFU were determined by plating on Mueller-Hinton agar, while the viability curve was constructed by staining with 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC), which was used to determine the respiratory activity (hence viability) as described in Gangaiah et al. (2009) and elsewhere.

COVETED POTENTIALS: CONCLUSIONS AND CLOSING REMARKS

It has been reported that poly-P and cognate enzymes occur in many bacterial species, suggesting that poly-P metabolism might be ubiquitous in the prokaryotes (Kornberg et al., 1999; Rao et al., 2009). Further, many of these species have a confirmed ability to enter the VBNC state (**Table 2**), which increases the appeal of a possible VBNC–poly-P link as a potential universal denominator in the formation of this cellular state. While the latter needs further experimental proof, poly-P appears to be a contributor to VBNC formation in the unconventional *C. jejuni*. Subsequently, the bearings of the findings listed above on efforts aiming at reducing *C. jejuni* in the food chain might be important, because it is already known that *C. jejuni* VBNC can adhere to edible products (e.g., on the skin of chicken carcasses; Jang et al., 2007). Further, phosphate-containing chemicals and weak organic acids (e.g., acetate and formate) have either been typically used or investigated as means to process, preserve, or decontaminate foods (Sofos and Smith, 1998; Capita et al., 2002; Hirshfield et al., 2003; Ricke, 2003). Organic acids, including formic acid, have also been used or tested as potential feed additives to reduce food-borne pathogens in animals including chickens, the primary reservoir of *C. jejuni*

Table 2 | A list of food-borne bacterial pathogens that possess homologs of the PPK enzymes (Rao et al., 2009).

VBNC-forming food-borne bacteria	Polyphosphate kinase 1 (PPK1) and PPK2 homologs
Campylobacter jejuni	PPK1, PPK2
Campylobacter coli	PPK1, PPK2
Enterococcus faecalis	PPK1
Escherichia coli	PPK1, PPK2
Helicobacter pylori	PPK1
Salmonella enterica serovar, Enteritidis	PPK1
S. Typhimurium	PPK1
Shigella dysenteriae type 1	PPK1
Shigella flexneri	PPK1
Vibrio cholerae	PPK1, PPK2
Vibrio parahaemolyticus	PPK1, PPK2
Yersinia enterocolitica	PPK1

These species/strains also have a documented ability to form VBNC (Rowan, 2004).

(Ricke, 2003). However, these chemicals might be inducers or modulators for the food-borne pathogen to enter the difficult-to-detect VBNC state.

Admittedly, the role of VBNC in the spread of food-related infections is not clear; however, it can be argued that caution is warranted in scenarios that involve food safety. Therefore, investigations on VBNC regarding (a) factors that trigger their formation, (b) mechanisms of their formation, (c) their virulence properties, and (d) conditions that favor their infectivity are important for proper assessment of the impact of VBNC on food safety and public health.

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Compartment-specific pH monitoring in *Bacillus subtilis* using fluorescent sensor proteins: a tool to analyze the antibacterial effect of weak organic acids

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The internal pH (pH_i) of a living cell is one of its most important physiological parameters. To monitor the pH inside *Bacillus subtilis* during various stages of its life cycle, we constructed an improved version (IpHluorin) of the ratiometric, pH-sensitive fluorescent protein pHluorin by extending it at the 5′ end with the first 24 bp of *comGA*. The new version, which showed an approximate 40% increase in fluorescence intensity, was expressed from developmental phase-specific, native promoters of *B. subtilis* that are specifically active during vegetative growth on glucose (P_{ptsG}) or during sporulation (P_{spoIIA}, $P_{spoIIID}$, and P_{sspE}). Our results show strong, compartment-specific expression of IpHluorin that allowed accurate pH_i measurements of live cultures during exponential growth, early and late sporulation, spore germination, and during subsequent spore outgrowth. Dormant spores were characterized by an pH_i of 6.0 ± 0.3. Upon full germination the pH_i rose dependent on the medium to 7.0–7.4. The presence of sorbic acid in the germination medium inhibited a rise in the intracellular pH of germinating spores and inhibited germination. Such effects were absent when acetic was added at identical concentrations.

Keywords: bacterial spore formers, spores, spore germination, intracellular pH, GFP, pHluorin, weak organic acids, uncouplers

INTRODUCTION

The internal pH (pH_i) of living cells plays a fundamental role in many chemical reactions. Many intracellular enzymes show optimal activity and stability in a narrow pH range near neutrality. Furthermore, in many organisms proton gradients are required for the greater part of ATP synthesis while uptake systems often depend on the proton gradient over the cell membrane (Krulwich et al., 1998, 2011; Slonczewski et al., 2009). In the model eukaryote *Saccharomyces cerevisiae*, pH_i was found to be a signal controlling growth (Orij et al., 2011). Gene expression as a response to glucose starvation was found to be mediated by changes in the pH_i, through the protonation state-dependent binding of a transcription factor to membrane-associated phosphatidic acid (Young et al., 2010). In multicellular eukaryotes pH_i is thought to be important during growth and differentiation (Cruciat et al., 2010). In prokaryotic organisms, the relationships between pH_i and growth and development have not been studied extensively (Padan and Schuldiner, 1987).

Because of its various well-described differentiation modes, *B. subtilis* is generally considered to be the bacterial model organism for cellular differentiation. The best described mode of differentiation of this Gram-positive prokaryote is sporulation, with the pathways controlling sporulation understood in great molecular detail (Eichenberger et al., 2004; Steil et al., 2005; Wang et al., 2006). Germination is less well understood, but Keijser et al. (2007) have shown that this too, is a carefully orchestrated process. We reasoned that in analogy to eukaryotes, pH_i could be a global regulator, as well as an indicator of the metabolic and energetic state of the cell. To gain further insight in the putative pH_i dynamics of these differentiation processes, we studied the pH_i of the mother cell and fore-spore independently. During sporulation the development of the pH_i in the mother cell and the nascent fore-spore may also give insight in the level of independence of the two cells.

In *B. subtilis*, the pH_i of the developing pre-spore is generally assumed to drop to pH = 6.0–6.4 during sporulation (Magill et al., 1994). The drop in pH causes a decrease in activity of phosphoglycerate mutase (PGM), which catalyses the conversion of 3-phosphoglycerate (3-PGA) to 2-phosphoglycerate. The reduced activity of PGM causes the accumulation of 3-PGA in the pre-spore. *B. subtilis* spores are metabolically dormant and contain no measurable ATP or glucose that could act as energy source during spore germination (Singh et al., 1977; Magill et al., 1996). It is assumed that the accumulated 3-PGA serves as an initial carbon and energy source for the cell.

The cell's pH_i can be measured with various methods. Ideally, intracellular pH measurements should be conducted in systems containing Good's buffers (Good et al., 1966; Ferguson et al., 1980), to minimize the effect of the cell's surrounding (unless desired). The probe used to measure pH should maintain accuracy over the pH range assessed. In addition, both the presence of the probe itself in a cell as well as the detection method applied should have minimal effect on cell physiology. Currently used techniques include the distribution of radiolabeled membrane-permeant weak acids, ^{31}P nuclear magnetic resonance

(NMR), fluorescent dyes (e.g., carboxyfluorescein, carboxyfluorescein diacetate, and succinimidyl ester; Ugurbil et al., 1978; Booth, 1985; Bulthuis et al., 1993; Magill et al., 1994; Breeuwer et al., 1996; Leuschner and Lillford, 2000). These methods have the advantage that no genetic modification is required and in the case of fluorescent dyes, single cell measurements are possible (Slonczewski et al., 2009). Weak acid dyes or reporters may alter the pH_i and are therefore difficult to use accurately, and may require many treatment and incubation steps before measurement. ^{31}P NMR and radiolabeled compounds require extensive cell handling and high cell density, which also disturb cell physiology. Another useful method is the use of fluorescent proteins [green fluorescent protein (GFP) derivatives]. This does require the organism to be genetically accessible but allows direct, fast, and localized pH measurements. In our lab, we have successfully used ratiometric pHluorin (Miesenböck et al., 1998) for a number of years in *S. cerevisiae* (Orij et al., 2011; Ullah et al., 2012), and more recently also in *B. subtilis* (Ter Beek, 2009). However, the codon usage of pHluorin was not optimized for use in *B. subtilis*. Our initial experiments suggested that our results might benefit from an increase in fluorescence intensity. This might be achieved by improving translation initiation (Veening et al., 2004). We therefore fused the first eight amino acids of *comGA* to pHluorin (Veening et al., 2004), as this was shown to improve the signal strength of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). The pH-dependent ratiometric fluorescent properties of IpHluorin were not affected by this fusion. To monitor the pH_i of both the mother cell and the pre-spore, ratiometric GFP-based IpHluorin was expressed from a number of developmental phase-specific native promoters of *B. subtilis* (Hilbert, 2004).

Expression of IpHluorin resulted in strong, compartmentalized, and cell type-specific signals. This allowed us to monitor the pH_i during growth and sporulation, in both pre-spore, mother cell and mature spore, as well as during spore germination. Effects of the addition of sorbic and acetic acid on the pH_i of germinating spores are described.

MATERIALS AND METHODS

STRAINS AND GROWTH CONDITIONS

For general purpose growth, *Escherichia coli* MC1061 and *B. subtilis* PB2 strains were grown in Lysogeny broth (LB). For fluorescence measurements, *B. subtilis* strains were grown in defined liquid medium (M3G; Keijser et al., 2007) buffered at pH = 5.5 or 6.4 with 80 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), or at pH = 7.0 or 7.4 with 80 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS). All cultures were grown at 37°C, under continuous agitation at 200 rpm. When required, the following antibiotics were added: kanamycin for strains carrying pDG148-derived plasmids; 10 μg/ml for *B. subtilis* strains, 50 μg/ml for *E. coli* strains, spectinomycin for strains carrying pSG1729-derived plasmids or genomic inserts (50 μg/ml). The strains used in this study are listed in **Table 1**.

SPORULATION OF *B. subtilis* STRAINS

Spores of *B. subtilis* were prepared by glucose depletion of defined liquid medium (M3S, which is M3G without sodium glutamate), at pH = 7.0. Cultures were incubated for 4 days at 37°C under continuous agitation (200 rpm). Spores were harvested and purified by extensive washing with MilliQ water at 4°C. The spore crops were inspected by phase-contrast microscopy and were free (>99%) of vegetative cells, germinating spores, and debris. Spores were stored for up to 1 week in MilliQ water at 4°C at optical density $(OD)_{600} = 1$.

CLONING OF PROMOTER FUSIONS WITH *IpHluorin*

Our initial experiments suggested that the accuracy of pH measurements might benefit from increased expression of pHluorin. To improve translation efficiency, the first 24 bp of *comGA*, with an ATG start codon, were fused to *pHluorin* by a polymerase chain reaction (PCR) with Pfu polymerase using primers IpHlu_2010_FW and IpHlu_2010_RV. This sequence was subsequently extended with a standard Shine–Dalgarno (SD) region (AAGGAGGAAGCAGGT; Joseph et al., 2001) using primers IpHlu_pDGA_FW. This SD-improved *pHluorin* (*IpHluorin*) was

Table 1 | Strains used in this study.

Strains	Genotype	Reference or construction
E. coli MC1061	Cloning host; F$^-$ *araD139 (ara-leu)7696 (lac)X74 galU galK hsdR2 mcrA mcrB1 rspL*	Casadaban and Cohen (1980)
Bacillus subtilis		
PB2	*trp*2C; 168 wild-type	C.W. Price
PB2 pDG148	*trp*2C; pDG148	This work
PB2 Pxyl-pHluorin	*trp*2C; *amyE3′ spc Pxyl–pHluorin amyE5′*	This work
PB2 Pxyl-IpHluorin	*trp*2C; *amyE3′ spc Pxyl–IpHluorin amyE5′*	This work
PB2 pDG-pHluorin	*trp*2C; pDG-pHluorin	This work
PB2 pDG-IpHluorin	*trp*2C; pDG-IpHluorin	This work
PB2 PptsG-IpHluorin	*trp*2C; *amyE3′ spc PptsG–IpHluorin amyE5′*	This work
PB2 PspoIIA-IpHluorin	*trp*2C; *amyE3′ spc PspoIIA–IpHluorin amyE5′*	This work
PB2 PspoIIID-IpHluorin	*trp*2C; *amyE3′ spc PspoIIID–IpHluorin amyE5′*	This work
PB2 PsspE-IpHluorin	*trp*2C; *amyE3′ spc PsspE–IpHluorin amyE5′*	This work

inserted between the *Hin*dIII and *Sal*I sites of pDG148. This construct, pDG-IpHluorin, was transformed into *B. subtilis* PB2 and compared with PB2 carrying pDG-pHluorin to analyze expression levels and pH-dependent characteristics of pHluorin and IpHluorin. Also, a xylose-inducible, genome-integrated expression system was constructed. To this end, *IpHluorin* was inserted in pSG1729, between the *Avr*II and *Hin*dIII sites, thereby replacing GFP and placing *IpHluorin* under control of the xylose-inducible P_{xyl} promoter.

To monitor the pH_i of *B. subtilis* for extended periods of time in different phases of its life cycle, without the need for externally supplied expression inducers, the promoter region of several growth phase-specific genes (P_{ptsG}, for vegetative cells growing on glucose, P_{spoIIA}, specific for pre-septum, sporulating cells, P_{sspE}, a fore-spore-specific gene, and $P_{spoIIID}$, a mother cell-specific promoter) of *B. subtilis* were selected for their expression levels (Steil et al., 2005; Veening et al., 2006a). Approximately 500 bp upstream of the start codon were selected for cloning. By standardizing the SD region, we aimed to increase and standardize the expression levels of poorer promoter sites (Ozbudak et al., 2002; Botella et al., 2010). The promoter and *SD-IpHluorin* sequences were fused by a PCR and inserted in pSG1729, between the *Avr*II and *Hin*dIII sites, thereby replacing the GFP and placing IpHluorin under control of a *B. subtilis* promoter. All enzymes used were obtained from Fermentas (Thermo Fisher Scientific).

Bacillus subtilis PB2 was used as target for our transformations. *B. subtilis* cells were made transformation-competent as described before (Kunst and Rapoport, 1995). The newly constructed plasmids were integrated in the *amyE* locus as described (Lewis and Marston, 1999). All plasmids and oligonucleotides used in this study are listed in **Tables 2** and **3**.

CALIBRATION OF *IpHluorin*

Bacillus subtilis PB2 containing either pDG148, pDG-pHluorin or pDG-IpHluorin were grown to exponential phase in M3G at pH 7.0 containing 10 μg/ml kanamycin. Bacterial growth and expression levels of ratiometric pHluorin and IpHluorin were monitored in a FluoStar Optima (BMG Labtech, Germany) for 3 h after addition of 0–1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

For calibration of the pH, expression of ratiometric pHluorin and IpHluorin was induced for 2.5 h by the addition of 1 mM IPTG. At $OD_{600} = 0.4$ the cells were centrifuged and resuspended in buffers with pH values ranging from 5.0 to 8.5 prepared from 0.1 M citric acid and 0.2 M K_2HPO_4. The intracellular and extracellular pH were equilibrated by the addition of 1 μM valinomycin and 1 μM nigericin (Breeuwer et al., 1996). Cells were transferred to black-walled microtiter plates and incubated at 37°C in a FluoStar Optima. OD_{600} was measured before start of the experiment. The ratio of emission intensity at 510 nm resulting from excitation at 390 and 470 nm (with photomultiplier gain set to 2,000) was calculated as described previously (Orij et al., 2011). Fluorescence and OD_{600} were monitored for 30 min, with measurements taken every 5 min. Calibration curves for pHluorin and IpHluorin were identical, with only minor fluctuations in fluorescence in time observed with pHluorin at pH = 8.5. From this, we concluded that the intracellular and extracellular pH had equilibrated rapidly. *B. subtilis* PB2 carrying pDG148 was measured for background fluorescence. Background fluorescence was subtracted at individual wavelengths before calculating the ratio. The calibration curve was determined by fitting the data of three independent biological replicates, each consisting of three technical replicates, with a polynomial curve of the third order.

BATCH MEASUREMENTS OF pH_i DURING SPORULATION, GERMINATION, AND OUTGROWTH

To monitor pH_i during growth and sporulation, all *B. subtilis* strains, wild-type (WT) (PB2) and those with IpHluorin fused to endogenous promoters were grown as described, in M3S without antibiotics, pH 7.0, to an $OD_{600} = 0.1$ in an incubator at 37°C under continuous agitation (200 rpm). Cell suspensions were diluted twofold by adding 50 μl of culture to 50 μl of medium in black microtiter plates which were then monitored in a FluoroStar Optima BMG (Labtech, Germany) at 37°C. OD_{600} and pH measurements were taken every 10 min for 48 h. The plates were shaken (200 rpm) in between measurements thus ensuring optimal growth (Ter Beek, 2009). For spore germination, washed spores were heat activated (30 min, 70°C, then cooled on ice) and subsequently mixed 1:1 with 2× concentrated M3 with or without glucose, containing weak organic acid (WOA) in predetermined

Table 2 | Plasmids used in this study.

Plasmid	Genotype	Reference or construction
pDG148	*bla ble kan lacI Pspac*	Stragier et al. (1988)
pDG-pHluorin	(pDG148); *pHluorin*	
pDG-IpHluorin	(pDG148); *IpHluorin*	This work
pSG1729	*bla amyE3′ spc Pxyl–gfpmut1′ amyE5′*	Lewis and Marston (1999)
pSG-pHluorin	*bla amyE3′ spc Pxyl–pHluorin amyE5′*	This work
pSG-IpHluorin	*bla amyE3′ spc Pxyl–IpHluorin amyE5′*	This work
$pSGP_{ptsG}$-IpHluorin	*bla amyE3′ spc PptsG–IpHluorin amyE5′*	This work
$pSGP_{spoIIA}$-IpHluorin	*bla amyE3′ spc PspoIIA–IpHluorin amyE5′*	This work
$pSGP_{spoIIID}$-IpHluorin	*bla amyE3′ spc PspoIIID–IpHluorin amyE5′*	This work
$pSGP_{sspE}$-IpHluorin	*bla amyE3′ spc PsspE–IpHluorin amyE5′*	This work

Table 3 | Oligonucleotides used in this study.

Oligonucleotide	Sequence (5′–3′)	Remarks
IpHlu2010_FW	ATGGATTCAATAGAAAAGGTAAGC**ATG**AGTAAAGGAGAAGAAC	Forward primer for *IpHluorin*
IpHlu148_RV	CGACGTCGACTTTATTTGTATAGTTCATCCATGCC	Reverse *IpHluorin* primer for pDG148
IpHlu148A_FW	CCCAAGCTTAA*GGAGGA*AGCAGGT**ATG**GATTCAATAGAAAAG	Forward *IpHluorin* primer for pDG148
IpHlu1729A_FW	ACGCCCTAGG**ATG**GATTCAATAGAAAAGGTAAGC	Forward *IpHluorin* primer for pSG1729
IpHlu2010_RV	CCCAAGCTTTTATTTGTATAGTTCATCCATGCCATG	Reverse *IpHluorin* primer for pSG1729
PptsG_FW	ACGCCCTAGGGAAAGTAAATAAGGAAAGTGTCAAC	5′ end of P_{ptsG}
PptsG_IpHlu_RV	CATACCTGCTT*CCTCCT*TTTTTACTAGTCTGACCTTAC	3′ end of P_{ptsG}
PptsG_IpHlu_FW	GTAAGGTCAGACTAGTAAAAA*AGGAGGA*AGCAGGT**ATG**	3′ of P_{ptsG} and 5′ of *IpHluorin*
PspoIIAA_FW	ACGCCCTAGGCCATAGCGGTTGTATTC	5′ end of P_{spoIIA}
PspoIIAA_IpHlu_RV	CATACCTGCTT*CCTCCT*TGATATGATCGGATAATGAGTGTTTC	3′ end of P_{spoIIA}
PspoIIAA_IpHlu_FW	GAAACACTCATTATCCGATCATATCA*AGGAGG*AAGCAGGT**ATG**	3′ of P_{ptsG} and 5′ of *IpHluorin*
PspoIIID_FW	ACGCCCTAGGCTGACCATTGAGATGAATAAAG	5′ end of $P_{spoIIID}$
PspoIIID_IpHlu_RV	**CAT**ACCTGCTT*CCTCCT*TAAAATGGATGTGAGAAGTGTGAAATGAG	3′ end of $P_{spoIIID}$ and 5′ of IpHluorin
PspoIIID_IpHlu_FW	CTCATTTCACACTTCTCACATCCATTTTA*AGGAGG*AAGCAGGT**ATG**	3′ of P_{ptsG} and 5′ of *IpHluorin*
PsspE_FW	ACGCCCTAGGTGAACATTAATGCGAAAGCATTG	5′ end of P_{sspE}
PsspE_IpHlu_RV	**CAT**ACCTGCTT*CCTCCT*TCGGTCATTAGAATGTCCAG	3′ end of P_{sspE}
PsspE_IpHlu_FW	CTGGACATTCTAATGACCGA*AGGAGG*AAGCAGGT**ATG**	3′ of P_{sspE} and 5′ of *IpHluorin*

Underlined bases: restriction enzyme recognition sites; bases in italics: Shine–Dalgarno sequence; bold bases: start codon.

concentrations. To trigger germination, 5 µl 20× concentrated AGFK (10 mM L-asparagine, 10 mM D-glucose, 1 mM D-fructose, 1 mM KCl; Wax and Freese, 1968) was added. Microtiter plates were placed in a FluoStar Optima (BMG Labtech, Germany) at 37°C and shaken between measurements (200 rpm). Growth was monitored for 2–12 h, with pH and OD_{600} measurements taken every 10 min.

MICROSCOPY

To verify if expression of IpHluorin was correctly localized, *B. subtilis* cells were cultured as described above for batch measurements at pH = 7.0. All strains were grown as described to exponential phase or for 16–24 h to observe sporulating cells. Cells were immobilized on 1% agarose (Koppelman et al., 2004), and photographed with a CoolSnap *fx* (Photometrics) charge-coupled device (CCD) camera mounted on an Olympus BX-60 fluorescence microscope through an UPLANFl 100×/1.3 oil objective (Japan) with a 41017 - Endow GFP/EGFP Bandpass filter (Chroma Technology Corp., Bellows Falls, VT, USA).

RESULTS

IMPROVED EXPRESSION OF pHluorin

Many microorganisms have an internal (cytosolic and/or mitochondrial) pH between 7 and 8 (Orij et al., 2009; Slonczewski et al., 2009) during optimal growth and maintaining pH homeostasis is of vital importance for most, including *B. subtilis* where pH_i differences have been inferred for its various developmental phases. We now used the pH-sensitive GFP pHluorin, developed for yeast (Miesenböck et al., 1998), to directly measure on-line the pH_i dynamics in *B. subtilis*. Codon usage of this GFP was not optimized for *B. subtilis* and our initial experiments suggested that expression might be improved. It was shown previously that addition of the first eight amino acids of *comGA* improved translation initiation efficiency of CFP and YFP in *B. subtilis* (Veening et al., 2004). We used this approach to construct improved pHluorin (IpHluorin; **Figure 1A**).

To analyze fluorescence intensity of *Bacillus* cells harboring pDG148, pDG-pHluorin, or pDG-IpHluorin cells were transferred to microtiter plates containing 0–1 mM IPTG to induce expression. Cell growth was monitored for 3 h, together with fluorescence emission at 510 nm upon excitation at 390 and 470 nm. The three strains compared had identical growth rates (not shown). Fluorescence intensity after 2.5 h is shown in **Figure 1B**, and depended on the concentration of IPTG. Cells expressing IpHluorin had the highest signal intensity at 1 mM IPTG, approximately 40% higher than pHluorin under the same conditions. The most important characteristic of pHluorin is its shift in excitation wavelength depending on the pH of its surroundings. To establish if the addition of eight amino acids at the N-terminus would alter these characteristics, calibration curves for pHluorin and IpHluorin were made (**Figure 1C** for IpHluorin, not shown for pHluorin). Both growth rate and the ratiometric characteristics were similar and allowed reliable pH_i readings in a range between pH 5 and 8.5 in live *B. subtilis* cultures.

THE INTERNAL pH VARIES WITH GROWTH PHASE

The cytosolic pH is a crucial parameter for bacteria because it modulates the activity of many enzymes (Vojinovic and Von Stockar, 2009) and in many species plays a crucial role in generating the

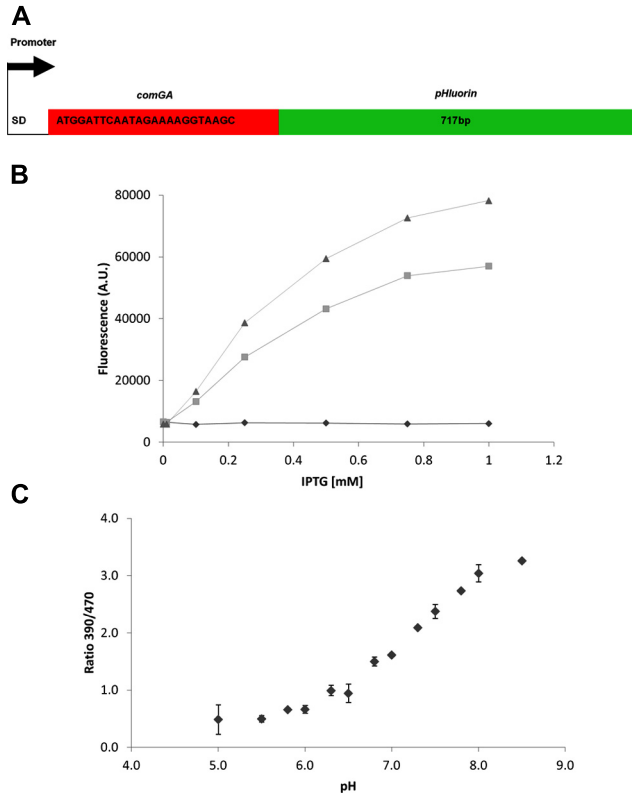

FIGURE 1 | Improved expression of pHluorin in *B. subtilis*. **(A)** Schematic overview of the improved pHluorin construct. SD – Shine–Dalgarno sequence. comGA – the first 24 bp of *comGA*, with the start codon converted to ATG. **(B)** Expression levels of pHluorin and improved pHluorin after induction with IPTG. Fluorescence (AU, arbitrary units) at 510 nm from excitation at 390 nm was measured after 2.5 h of induction and normalized to OD_{600}. Diamonds, control (pDG148); squares, pHluorin (pDG-pHluorin); triangles, IpHluorin (pDG-IpHluorin). **(C)** Calibration curve for IpHluorin. Error bars indicate standard deviations ($n = 3$).

Table 4 | Promoters used for IpHluorin expression.

Promoter	Regulator	Corresponding growth phase
P_{ptsG}	σ^A	Growth on glucose (Botella et al., 2010)
P_{spoIIA}	Spo0A, $\sigma^{F,G,H}$	Early sporulation (Wang et al., 2006)
$P_{spoIIID}$	σ^E	Early sporulation, mother cell-specific (Wang et al., 2006)
P_{sspE}	σ^G	Late sporulation, spore-specific (Wang et al., 2006)

proton-motive force (Shioi et al., 1980; Slonczewski et al., 2009). To monitor pH_i during various stages of growth in *Bacillus*, we fused promoters of strongly expressed, growth phase-specific genes to IpHluorin. This allowed us to measure pH_i of *B. subtilis* without addition of inducers such as IPTG or xylose (**Figures 2A–H**). The selected promoters and their specific expression phase are shown in **Table 4**.

To monitor the pH_i during growth in minimal medium with glucose as the only carbon source, we used the promoter of *ptsG*, which encodes the glucose-specific enzyme II of the carbohydrate:phosphotransferase system to drive *IpHluorin* expression. P_{ptsG} is a strong promoter during vegetative growth on glucose (Botella et al., 2010). Expression of IpHluorin from the P_{ptsG} promoter follows the growth curve closely (**Figure 3A**). When the cells die or move into stationary phase (after 7.5 h), the signal intensity remains high and stable. The sporulation-specific promoters (**Figure 4B**) are activated after the drop in OD_{600}, signifying the onset of sporulation.

The pH_i of *B. subtilis* reaches its highest value of around 8 during exponential growth. This value is in agreement with earlier reported values ranging from pH = 7.8 to 8.1 (Setlow and Setlow, 1980; Magill et al., 1994). At the drop in OD_{600}, cells either die or differentiate and initiate sporulation or remain in stationary phase. This was accompanied by an apparent steep decrease in pH_i, to 7.0 in vegetative cells expressing IpHluorin from P_{ptsG}. Likely, this at least partially is indicative for cell lysis as a strong fluorescent signal could also be detected in the medium after spinning down the cells. Additionally, it is possible that morphological changes of the cell affect their optical properties. Sporulating cells are, for instance, smaller than exponentially growing cells. Hence, after sporulation commences, the pH values observed with P_{ptsG}-IpHluorin can no longer be considered an accurate estimate of the intracellular pH in vegetative cells. Apart from aberrant values due to cell lysis, the P_{ptsG}-driven IpHluorin may also get trapped in sporulating cells so that the observed pH from P_{ptsG}-driven IpHluorin is the average of sporulating and non-sporulating cells as well as the medium. Subsequently, the OD_{600} rose again slowly and the apparent pH increased to 7.4 (**Figure 3**). We do not know from which cells this signal originates as it may represent the average of various differentiation types, all expressing IpHluorin. To deconvolute these signals, single cell measurements are needed.

SPORULATION-SPECIFIC EXPRESSION OF IpHluorin

Sporulation of *B. subtilis* is a well-described, carefully orchestrated process where a number of different sigma factors are activated during subsequent stages (Wang et al., 2006). It has been reported that the pH_i of *Bacillus* spores is lower than that of vegetative cells (Magill et al., 1994). We set out to measure the pH in spores and at what stage in sporulation the drop in pH starts and when the pH would rise again during germination. For this purpose, we constructed strains with early and late (pre)spore-specific expression of IpHluorin. We selected promoters that would be active in the pre-spore and mother cell at different times during sporulation to monitor pH_i of both cells separately. Expression from P_{spoIIA}, $P_{spoIIID}$, and P_{sspE} starts after the drop in OD_{600}. The surviving cells may prepare for diauxic growth or sporulation (Veening et al., 2008). This characteristic allowed us to measure differences in pH_i in both mother cell and pre-spore in the subpopulation that initiates sporulation. Expression levels from P_{spoIIA}, $P_{spoIIID}$, and P_{sspE} are lower than of P_{ptsG}, but are still reliable and strong enough to allow pH monitoring (**Figures 3A,B**). For the sporulation-specific promoters, a cut-off of 1,000 arbitrary units in the 390 to 510 nm fluorescence channel was used for pH_i calculations.

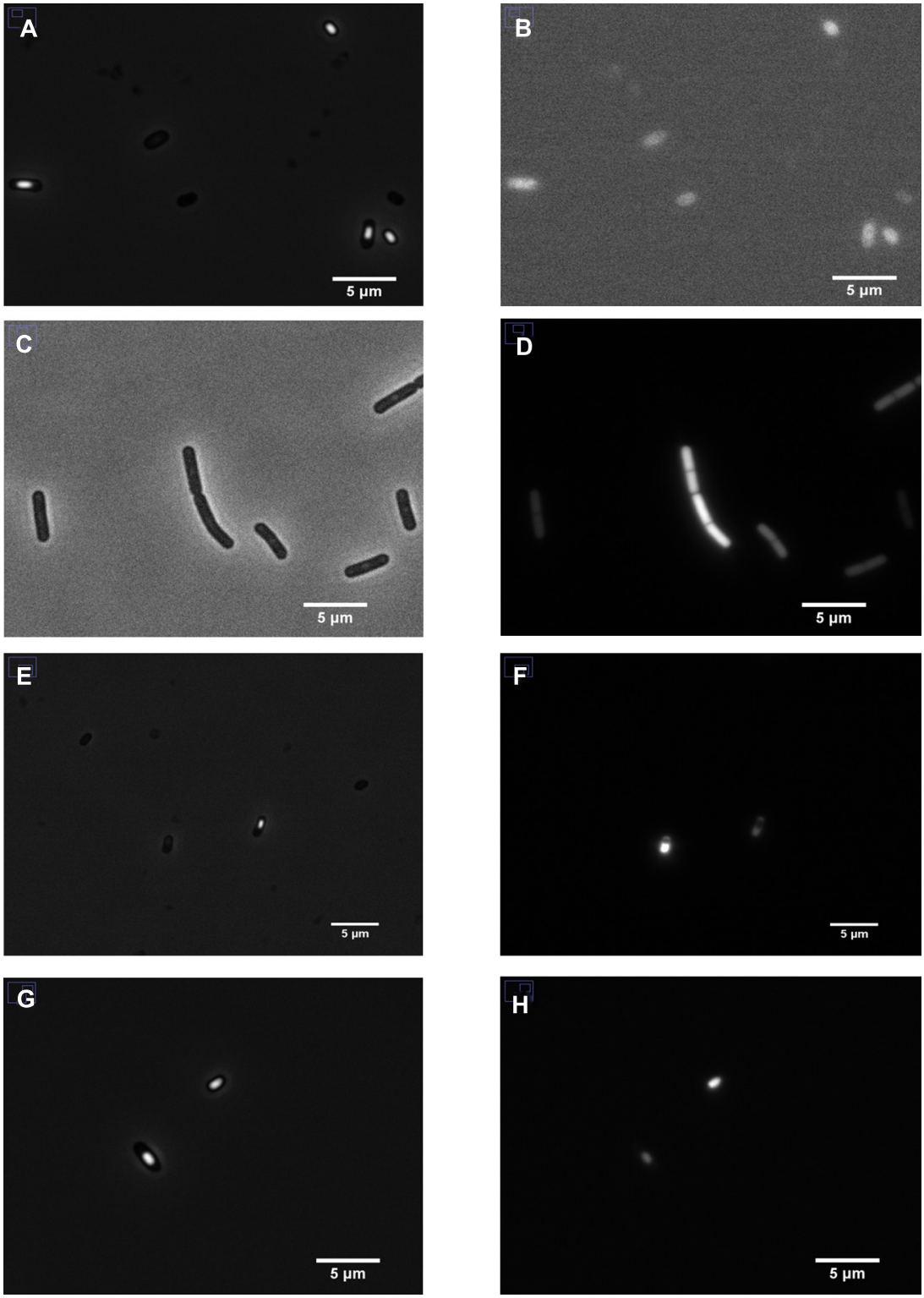

FIGURE 2 | Expression of lpHluorin in *B. subtilis* PB2. *Left panels*: phase-contrast images; *right panels*: corresponding fluorescent signals. **(A,B)** Non-transformed cells; **(C,D)** PptsG-lpHluorin-expressing cells; **(E,F)** sporulating cells expressing PspoIID-lpHluorin; **(G,H)** sporulating cells expressing PB2 PsspE-lpHluorin.

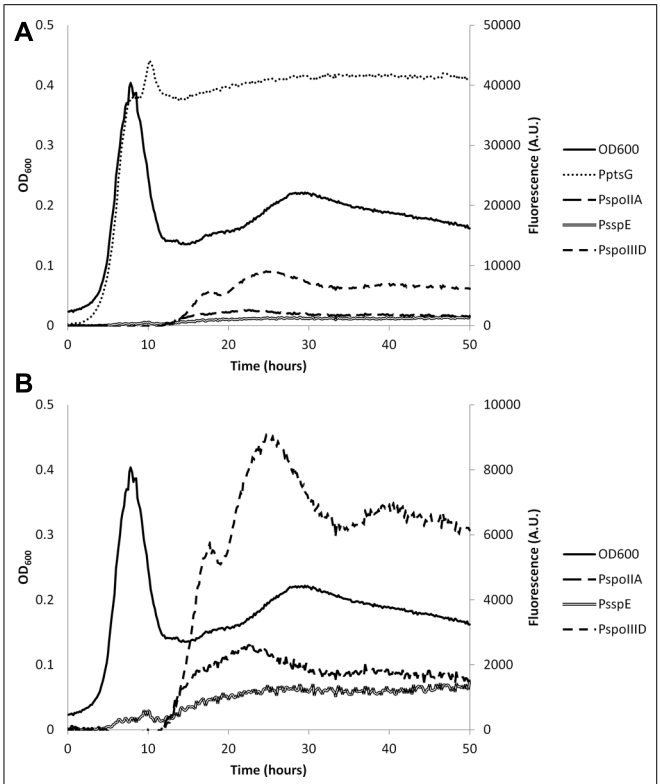

FIGURE 3 | Representative example of the expression levels of lpHluorin from different promoters during different growth stages. **(A)** Fluorescence levels from excitation at 390 nm and emission at 510 nm from different promoters used. **(B)** Identical to **(A)**, but without P_{ptsG}-lpHluorin. Expression of lpHluorin from sporulation-specific promoters starts only after the drop in OD_{600}.

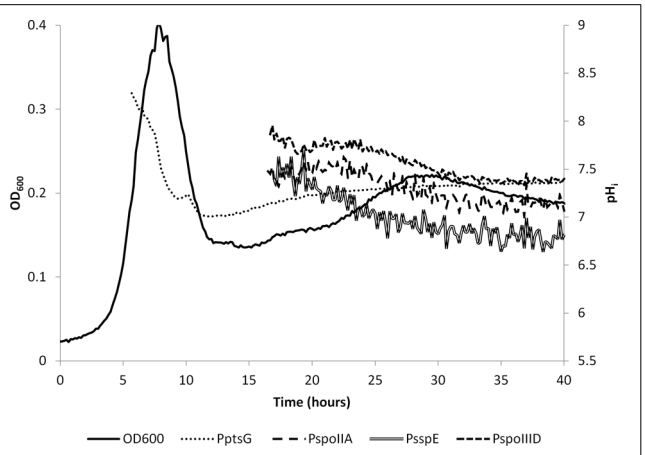

FIGURE 4 | Internal pH as measured with lpHluorin expressed from different promoters. The pH is calculated from when the 390 nm channel exceeded 1,000 units. pH from the spore-specific promoters is calculated from approximately 17 h onward. Lines represent actual data from a representative example.

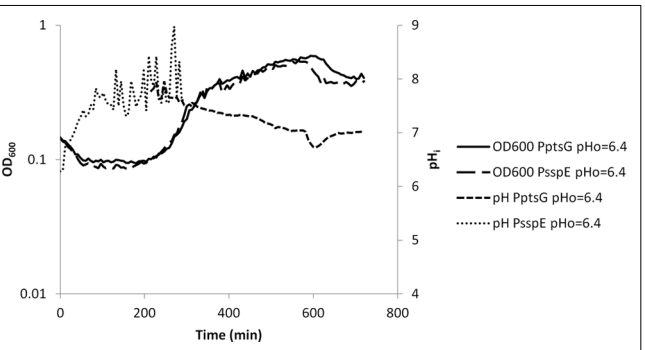

FIGURE 5 | Internal pH during spore germination and outgrowth. lpHluorin accumulated in the spores (from expression controlled by P_{sspE}) allows pH measurements from $t = 0$ to approximately 300 min. Expression of lpHluorin from P_{ptsG} allows calculation of the pHi from approximately 200 min. Data are from a representative example of germination and outgrowth of *B. subtilis* spores at an external pH of 6.4.

SpoIIA is activated by high levels of activated Spo0A and its presence was shown to be a reliable indicator for cells that initiate sporulation (Veening et al., 2005). Indeed, a fluorescent signal of a GFP reporter under control of the SpoIIA promoter can be found in both mother cell and fore-spore (our unpublished observations; Veening et al., 2006b). Expression of genes in the mother cell regulated by $P_{spoIIID}$ follows that of those regulated by P_{spoIIA}, as expected, but because expression levels of $P_{spoIIID}$-controlled lpHluorin are higher, reliable pH_i measurements can be obtained earlier with the latter. Initially, the sporulating subpopulation had a pH_i that closely resembled the pH_i of exponentially growing cells measured with the P_{ptsG}-lpHluorin strain. The mother cell ($P_{spoIIID}$-lpHluorin) had a pH of 7.8 after 17 h of incubation. lpHluorin expressed from the spore-specific promoter P_{sspE} revealed pH values of 7.4. The mother cell-specific expression of lpHluorin from the *spoIIID* promoter decreased after 25 h of culture. At that time point and from then onward, an apparent decrease measured with the mother cell-specific promoter driving lpHluorin expression was observed. This data, however, may at least partially be influenced by mother cell lysis and release of lpHluorin into the medium. The inferred pH at 40 h of culture closely resembled medium pH, corroborating this notion.

The decrease in pH_i in the fore-spore (P_{sspE}-lpHluorin) drops below the medium pH and its fluorescent signal can clearly be observed inside maturing spores (**Figure 2H**). Noticeably, spores have a very low water activity and optical properties dissimilar from vegetative cells, which may obscure the pH as defined as the number of free protons (Sunde et al., 2009).

Our data indicates that at 17.5 h of culture, the pH_i of the fore-spore is 7.4, as reported by P_{sspE}-lpHluorin. After 40 h, the pH value of 6.8 reported by P_{sspE}-lpHluorin is approaching the reported value for *Bacillus* spores ($pH_i = 6.0 \pm 0.3$; Barton et al., 1980; Setlow and Setlow, 1980; Magill et al., 1994, 1996). Likely, because at this time point the population is still a mix of some fore-spore-containing cells as well as many free spores, the observed pH is slightly higher than the reported values for isolated *Bacillus* spores. Corroborating this, when we washed and isolated the spores our pHluorin-based measurement of the pH_i of *B. subtilis* spores also indicated values around 6.0 ± 0.3 (see, e.g., pHi data of time point 0 obtained with lpHluorin driven by P_{sspE} in **Figure 5** and beyond).

INTERNAL pH DURING SPORE GERMINATION AND OUTGROWTH

As described above, IpHluorin expressed from P_{sspE} accumulates in mature spores. Germination and outgrowth were monitored used *B. subtilis* PB2 P_{sspE}-IpHluorin and *B. subtilis* PB2 P_{ptsG}-IpHluorin. When germination is triggered by addition of a mixture of asparagine, glucose, fructose and potassium (AGFK), the OD_{600} of the spore crop drops, because the refractile spores turn phase-dark, due to water uptake. Simultaneously, the spore's pH_i rises. Depending on the medium pH, the pH_i rises to 7.0–7.4 (**Figure 5** and our unpublished observations for germination at pH 7.4, respectively). In the case of germination at pH = 6.4, this indicates the establishment of a pH gradient. Not all spores germinate at the same time, and significant heterogeneity can be observed in the timing of germination and outgrowth (Smelt et al., 2008). Since this is a mixed population, consisting of phase-bright and germinating spores, the actual pH change in individual germinating spores may differ.

During the lag phase between germination and outgrowth, the *ptsG* promoter is activated. Parallel expression of IpHluorin from this promoter shows that the pH measured this way lies between 7.5 and 7.8. This range of pH values is maintained during exponential growth. A generally observed slow decrease in pH may be due to acidification of the medium by acetic acid or CO_2 (Russell and Diez-Gonzalez, 1998; Orij, 2010). After approximately 600 min, there is a sudden drop in pH and OD_{600} as described above (**Figure 5**).

Germination with medium pH = 7.4 shows a more rapid decrease in OD_{600} and an equally faster rise in pH_i. Also, when outgrowth commences, pH_i of these cells is higher, but follows a similar trend as with medium at pH = 6.4.

INTERNAL pH DURING SPORE GERMINATION WITH WEAK ACID STRESS

Dormant spores are highly resistant to antimicrobial treatment, but also metabolically inert (Brul and Coote, 1999). When germination is triggered, the spore becomes more sensitive. Also, it has been observed that germination of *Bacillus* spores can be inhibited by various preservatives (Cortezzo et al., 2004; Van Melis et al., 2011). When spores start to germinate, they release protons and the pH_i rises. Also, during this stage water is taken up and metabolism should be restarted. These processes might be a target moment for WOAs to halt outgrowth of the germinating spore.

Acetic and sorbic acid are amongst the most commonly used food preservatives (Stratford et al., 2009; Ter Beek and Brul, 2010; Ullah et al., 2012). While both WOAs have a similar pK_a value, sorbic acid is clearly the more potent antimicrobial compound. We compared the effects of sorbic and acetic acid on germination and outgrowth by using concentrations of both acids that had a similar effect on growth rate (Ter Beek, 2009). Low concentrations of both acids reduced the exponential growth rate by approximately 50%. Spores germinating in medium (pH = 6.4) with 3 mM K-sorbate had a decreased rate of pH_i increase. In controls the pH_i increase between the start of germination and $t = 90$ min was 1.4 units whilst with 3 mM K-sorbate this was 0.7 units. At the onset of the exponential phase, the pH_i which gradually decreased from pH = 7.4 to 7.2 at $t = 11$ h (**Figure 6A**). Twenty-five millimolars of K-acetate allowed a rapid increase in pH_i during germination. The pH_i during exponential growth remained stable at 7.2 during the experiment (**Figure 7A**).

High concentrations of WOAs were selected to reduce growth by 85%. P_{ptsG}-driven expression of IpHluorin is delayed under these conditions, while spore-specific IpHluorin can be observed for longer periods of time because the signal is not diluted out. K-sorbate (10 mM) is shown to delay the maximum drop in OD_{600} indicative for spore germination. The data in **Figure 6B** show a drop from OD_{600} 0.13 to 0.08 in 216 min rather than from 0.13 to 0.07 in 84 min as was seen in the control shown in **Figure 5**. The rise of the pH_i was here similarly delayed as was the case with 3 mM K-sorbate. Such effects were not seen with 80 mM K-acetate, although the reduction in growth rate is similar (**Figure 7B**).

To further confirm the observation that sorbic acid inhibited the development of a positive inside pH gradient, spores of *B. subtilis* PB2 P_{sspE}-IpHluorin were incubated with identical concentrations of either sorbic or acetic acid in medium without glucose other than present as germinant. When germination was triggered by addition of AGFK, spores incubated with sorbic acid showed a clear concentration dependant reduction in OD_{600} drop-rate as well as a reduced pH_i increase-rate. The OD drop-rate decreased from 80×10^{-3} to 40×10^{-3} OD_{600}/min when 0.5 mM undissociated sorbic acid was present (**Figure 8B**). Such effects were not seen with acetic acid at identical concentrations, which

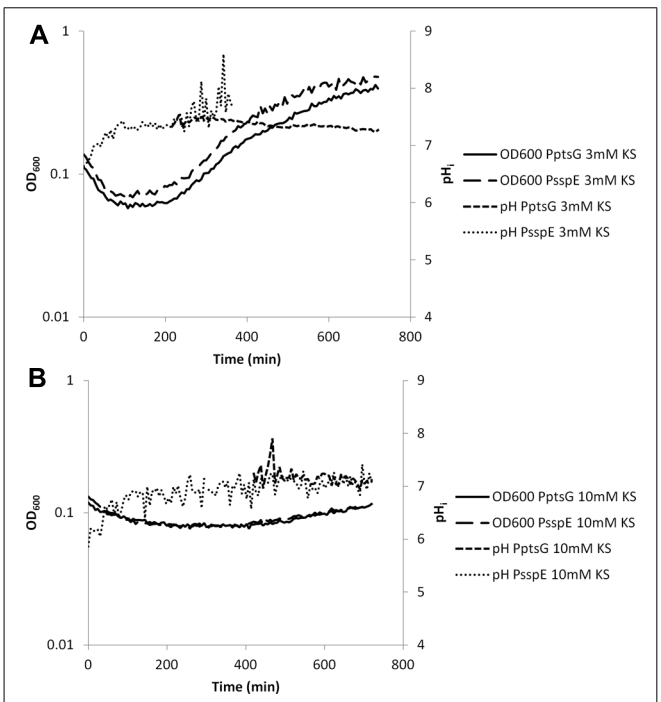

FIGURE 6 | Internal pH during spore germination and outgrowth. IpHluorin accumulated in the spores (from expression controlled by P_{sspE}) allows pH measurements from $t = 0$ to approximately 300 min. Expression of IpHluorin from P_{ptsG} allows calculation of the pHi from approximately 200 min. Data are from a representative example. **(A)** Germination and outgrowth of *B. subtilis* spores at an external pH = 6.4 with 3 mM KS. **(B)** Germination and outgrowth of *B. subtilis* spores at an external pH = 6.4 with 10 mM KS.

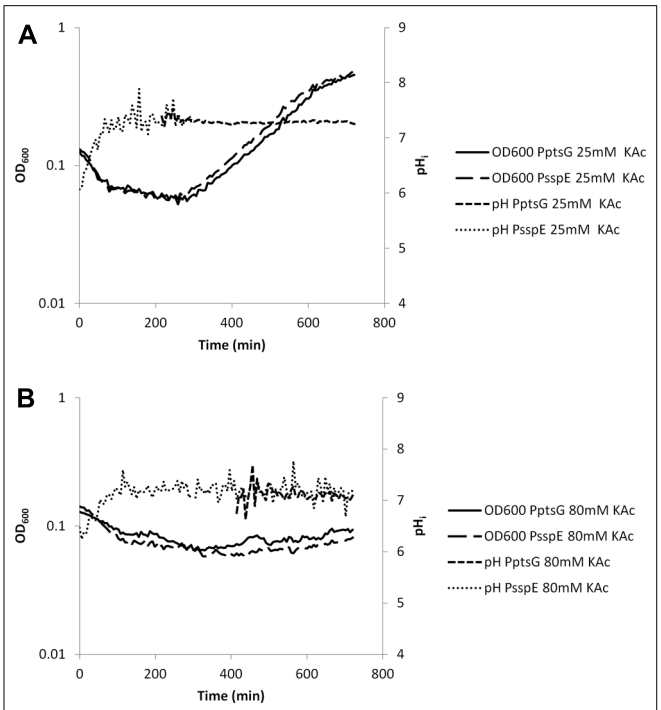

FIGURE 7 | Internal pH during spore germination and outgrowth. IpHluorin accumulated in the spores (from expression controlled by P_{sspE}) allows pH measurements from $t = 0$ to approximately 300 min. Expression of IpHluorin from P_{ptsG} allows calculation of the pHi from approximately 200 min. Data are from a representative example. **(A)** Germination and outgrowth of *B. subtilis* spores at an external pH = 6.4 with 25 mM KAc. **(B)** Germination and outgrowth of *B. subtilis* spores at an external pH = 6.4 with 80 mM KAc.

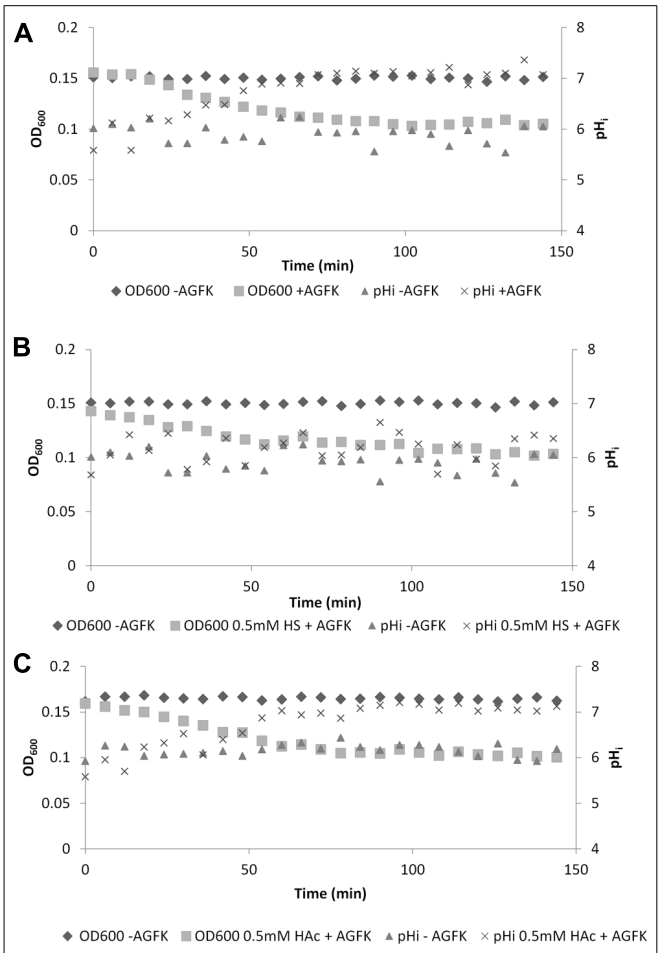

FIGURE 8 | OD_{600} and internal pH during germination of *B. subtilis* PB2 PsspE-IpHluorin spores in medium without glucose (pHo = 6.4). Data are from a representative example. **(A)** Germination with AGFK; **(B)** germination with AGFK in 0.5 mM sorbic acid; **(C)** germination with AGFK in 0.5 mM acetic acid.

behaved virtually identical to non-stressed germinating spores (**Figures 8A,C**). These observations are in agreement with earlier reports stating that sorbic acid can specifically inhibit germination of *B. cereus* and *B. subtilis*, likely by interacting with germinant receptors (Cortezzo et al., 2004; Van Melis et al., 2011).

DISCUSSION

We show here that IpHluorin is an accurate, versatile probe to investigate the pH_i of *B. subtilis*. We were able to improve expression of pHluorin by fusion of the first 24 bp of *comGA* with the pHluorin-encoding gene. Genomic integration of IpHluorin resulted in more homogeneous expression levels compared to a multi-copy plasmid. It also resulted in a more stable construct, not requiring antibiotics for maintenance of the IpHluorin gene during extended periods of growth (not shown). The use of genomically integrated constructs with endogenous promoters for the expression of IpHluorin resulted in a strong enough signal for accurate pH measurements during exponential growth on glucose as well as compartment-specific pH_i measurements during sporulation. The IpHluorin that accumulates in the spore under control of P_{sspE} allows pH_i measurements of the *B. subtilis* spore. During spore germination and outgrowth, the signal from IpHluorin, expressed from P_{sspE} overlaps slightly in time with P_{ptsG}-IpHluorin expression, thus allowing continuous pH_i monitoring during germination and outgrowth in batch. The pH values we have observed here closely resemble those found with other methods. During exponential growth, the pH_i approaches pH = 8. The pH_i of *B. subtilis* spores was also found to lie at approximately pH = 6. Despite the fact that expression levels of IpHluorin are much lower in spores, the pH value observed again closely corresponds to earlier reported values. The notion that during outgrowth a pH is observed that closely resembles the pH_i during exponential growth (as observed with P_{ptsG}-IpHluorin) further corroborates the accuracy of our method.

Other methods to measure pH_i generally involve compounds that are hydrophobic and have WOA groups and may act as uncouplers, thereby depleting the ΔpH and influencing $\Delta\Psi$ over the membrane. They are also more labor-intensive when high temporal resolution is required and except for fluorescent dyes do not allow cell type-specific pH measurements. However, these methods require long-term incubation with the dye plus extensive washing, taking up to 20 min to prepare the sample. Future studies will have to determine the phototoxicity and bleach rate of

IpHluorin in individual (growing, sporulating, and germinating) cells.

We have observed clear differences in pH_i between P_{ptsG}-IpHluorin and sporulation-specific IpHluorin. It has been shown that within a growing population of *B. subtilis* cells, differentiation occurs (Veening et al., 2006a,b) and this may affect metabolic state and pH_i. This heterogeneity cannot be clearly monitored in batch without the use of more specific promoters or single cell observations. Also during spore germination such heterogeneity is seen (Smelt et al., 2008), so our results show the average of a germinating population.

During spore germination, the pH_i increases due to release of protons (Swerdlow et al., 1981). This process follows the drop in OD_{600}, and results from H_2O uptake and release of DPA. Our results show that a ΔpH is established rapidly. Such an increased pH can reactivate PGM, thus allowing the utilization of the spore's 3-PGA store (Magill et al., 1994).

Taken together, our results show accurate, long-term pH_i monitoring in growing and sporulating *B. subtilis* cultures as well as during spore germination. The pH_i of sporulating cells is as high as that of exponentially growing cells. This is particularly the case for the mother cell. The pre-spore pH_i drops to pH = 6.0, however. The P_{ptsG}-IpHluorin strain can be used for many experiments where the pH_i needs to be measured in cells growing on glucose, without the need for additional inducers like IPTG. Also, antibiotics are not strictly necessary. The sporulation-specific IpHluorin-expressing strains may give more insight in compartmentalization during sporulation, while the P_{sspE}-IpHluorin strain may also help understanding spore germination characteristics in the presence of potential outgrowth inhibitors such as the WOAs sorbic acid and acetic acid.

Clearly, because not all cells are in exactly the same state, these data represent the average value of the pH_i in the population studies. To analyze the heterogeneity single-spore pH_i measurements are needed. Currently we are extending our single cell live imaging tool "SporeTracker" (Pandey et al., 2013) to that end.

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