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Comparative analysis of transcriptional and physiological responses of *Bacillus cereus* to organic and inorganic acid shocks

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Running title: Transcriptional and physiological responses of *B. cereus* to acid shocks

Keywords: Bacillus cereus; lactic acid; acetic acid; acid stress; microarrays; ATP-measurements

Abstract

Comparative phenotype and transcriptome analyses were performed with *Bacillus cereus* ATCC 14579 exposed to pH 5.5 set with different acidulants including hydrochloric acid (HCl), lactic acid (HL) and acetic acid (HAc). Phenotypes observed included a decreased growth rate (with HCl), bacteriostatic and bactericidal conditions, with 2 mM undissociated HAc or HL, and 15 mM undissociated HAc, respectively. In the latter condition a concomitant decrease in intracellular ATP levels was observed. The transcriptome analyses revealed general and specific responses to the acidulants used. The general acid stress response includes modulation of pyruvate metabolism with activation of the butanediol fermentation pathway, and an oxidative stress response that was, however, more extensive in the bacteriostatic and bactericidal conditions. HL-specific and HAc-specific responses include modulation of metabolic pathways for amino acid metabolism. Activation of lactate, formate, and ethanol fermentation pathways, alternative electron-transport chain components and fatty acid biosynthesis genes was noted in the presence of 15 mM undissociated HAc. In conclusion, our study has provided insights in phenotype-associated, and general and acidulant-specific responses in *B. cereus*.

1. Introduction

Bacillus cereus is a spore-forming Gram-positive bacterium that can cause diarrheic or emetic symptoms of food poisoning (Kotiranta *et al.*, 2000). The emetic syndrome is caused by consumption of cereulide-contaminated food, while the diarrhoeal syndrome is caused by enterotoxins that are produced by *B. cereus* cells in the small intestine (Stenfors Arnesen *et al.*, 2008). Food product groups that form a potential risk for *B. cereus* infections include ready-to-eat foods containing rice or pasta, milk and milk products, flavourings, pastry, vegetables and vegetable products (Wijnands *et al.*, 2006). *B. cereus* must be able to withstand low pH conditions such as encountered in foods acidified during food processing and conservation. Furthermore, enterotoxic *B. cereus* strains have to survive gastric transit to reach the human intestine. Therefore, a thorough understanding of the *B. cereus* response to acid shock may aid in defining safe food preservation conditions.

Organic acids like lactic acid (HL) and acetic acid (HAc) are often used as food preservatives. The pK_a of acids (3.86 for HL and 4.76 for HAc) determines the ratio between dissociated and undissociated forms at a given pH. The undissociated form can diffuse into the cell where it dissociates, releasing protons, until an equilibrium is reached. Different theories regarding the antimicrobial activity of organic acids have been raised, such as dissipation of the proton motive force, including lowering of the intracellular pH (pH_i), and intracellular accumulation of anions resulting in end-product inhibition (Brul and Coote, 1999; Cotter and Hill, 2003).

The responses of Gram-positive bacteria to acid stress are diverse (Cotter and Hill, 2003) and may include activation of proton pumps and protein repair systems, modification of cell membrane composition, production of alkali, and alteration of

metabolism. However, the acid stress response of *B. cereus* ATCC 14579 has not been studied extensively. Available information is limited to the acid tolerance response, which includes modulation of pH_i and protein synthesis (Browne and Dowds, 2002; Thomassin *et al.*, 2006). Moreover, most information on the bacterial acid stress response is related to acid shock treatment or acid tolerance resulting in inhibited growth, while there is no information available on the bacteriostatic and/or bactericidal response to acid stress, although this is most relevant for food preservation.

In this study, comparative phenotype and transcriptome analyses were performed with *Bacillus cereus* ATCC 14579 exposed to pH 5.5 set with different acidulants including hydrochloric acid (HCl), lactic acid (HL) and acetic acid (HAc). Phenotypic responses included decreased growth rates, bacteriostatic and bactericidal conditions, and these were linked with transcriptome analyses, providing insights in phenotype-associated, and general and acidulant-specific responses in *B. cereus*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacillus cereus type strain ATCC 14579 was obtained from the American Type Culture Collection and grown at 30ºC, 200 rpm in Brain Heart Infusion (BHI, Becton Dickinson, France) broth, buffered at pH 7.1 with 100 mM sodium phosphate. Cells were grown to exponential phase, at which the culture was acidified to pH 5.5. pH 5.5 acid shock was achieved by addition of 0.238% (v/v) 12 M HCl (Merck, Germany), by addition of 0.698% (v/v) HL (PURAC FCC 80; PURAC, the Netherlands), which

resulted in 2 mM undissociated HL, by addition of 0.205% (v/v) 12 M HCl in combination with 0.074% (v/v) HAc (Merck, Germany), which resulted in 2 mM undissociated HAc, or by addition of 0.571% (v/v) HAc, which resulted in 15 mM undissociated HAc. Impact of acid exposure on growth of *B. cereus* was assessed by measuring the optical density at 600 nm (OD, Novaspec II, Pharmacia Biotech, Germany) of the cultures at different time intervals. The survival upon acid shock was investigated by plating samples, taken at different time intervals, on BHI agar plates (15 g/l bacteriological agar, Oxoid, England) and overnight incubation at 30°C.

2.4. ATP measurements

The ATP concentration of samples obtained from the acid shocked cultures at different time intervals was measured. ATP measurements were performed using a Biocounter M2500 (Lumac BV, the Netherlands) in combination with the Microbial biomass kit (Celsis, the Netherlands) according to instructions of the manufacturer. The conversion of ATP to AMP by luciferase was measured in Relative Light Units (RLU). Using a range of ATP standards (100 nM – 100 μM), the unknown ATP concentration of the sample was determined. An integration and measuring period of 10 seconds was applied. Total ATP was measured by adding 2 ml absolute ethanol (Merck, Germany) to 1 ml of culture. After an incubation period of 10 minutes at -20ºC, 20 μl of the ethanol culture mixture was added to 180 μl of water and ATP was measured. ATP background levels were obtained by determining the ATP concentration of the supernatant.

2.2. RNA isolation, cDNA labelling and microarray hybridization

Samples for RNA isolation were taken at OD 0.5 just before addition of the acidulants and at 10, 30, and 60 min of exposure. Twenty ml of the culture was used for RNA isolation. After pelleting the cells in 30 sec (Eppendorf centrifuge 5804 R, Eppendorf, Germany), the supernatant was discarded and the cell pellets were resuspended in 1 ml Tri-reagent (Ambion, UK). The resuspended pellets were quick frozen in liquid nitrogen and stored at -80ºC until RNA isolation. RNA isolation, cDNA labelling and microarray hybridization were performed as described previously (van Schaik *et al.*, 2007). Custom-made Agilent *B. cereus* microarrays (GEO accession number GPL7679) were hybridized and after washing scanned in an Agilent microarray scanner (G2565BA). Data were extracted using Feature Extraction Software Version 8, which includes LOWESS normalization of the raw data.

2.3. Analysis of microarray data

After removal of the data for different controls printed on the microarray slides, the normalized data for each spot from the microarrays were analyzed for statistical significance using the Web-based VAMPIRE microarray suite (Hsiao *et al.*, 2005). A spot was found to be differentially expressed between two samples when the false discovery rate was smaller than 0.05. Subsequently, the data for the single spots were integrated to obtain expression ratios for an open reading frame. An open reading frame was found to be differentially expressed when all spots representing the open reading frame were significantly differentially expressed between the samples. The expression ratios of an open reading frame from duplicate experiments were averaged to obtain a single expression ratio per open reading frame. Finally, ratio changes of 2-fold (for up-regulated genes in the stress condition) and 0.5-fold (for down-regulated genes in the stress condition) were regarded as biologically significant (Wilks et al.,

2009). GeneMaths XT (version 1.6.1, Applied Maths, Belgium) was used for visualization, clustering and further analysis of the microarray data. The dendrogram of the microarray sets was generated using average linkage hierarchical clustering and the Euclidian distance matrix. Hierarchical clustering (complete linkage, Euclidian distance) of all genes was used to identify groups of genes with similar transcription profiles. The overrepresentation of functional classes within the groups of genes with similar expression profiles was evaluated using FIVA (Blom *et al.*, 2007) (results shown in *Supplementary material*).

3. Results

3.1. Growth and viability

The effects of pH 5.5 acid shock using different acidulants on *B. cereus* growth were determined (Fig. 1). pH 5.5 was set with HL and HAc, but because of their different pK_a this resulted in 2 and 15 mM undissociated acid, respectively. In addition, a combination of HAc and HCl was used to acidify the cultures to pH 5.5 to obtain a condition with 2 mM undissociated HAc. Adding HCl as acidulant had the mildest effect and led to a decreased growth rate compared to the untreated control. Addition of HL (2 mM undissociated acid) or HAc (2 mM or 15 mM undissociated acid) resulted in growth arrest. However, prolonged incubation revealed a clear difference between the conditions. In the conditions with 2 mM undissociated acids growth was resumed whereas the condition with 15 mM undissociated HAc did not show an increase of OD after 24 hours (data not shown). Assessment of colony forming units (cfu) after 24 hours showed the unstressed control cultures to have 9.2 ·

10^8 cfu/ml, HCl-shocked cultures to have $9.0 \cdot 10^8$ cfu/ml, the HL-shocked cultures $4.0 \cdot 10^8$ cfu/ml, the HAc/HCl-shocked cultures $2.6 \cdot 10^8$ cfu/ml, while the cfu of the 15 mM undissociated HAc shocked cultures were below the detection limit of 10^3 cfu/ml (data not shown). Assessment of the pH after 24 hours showed that the pH of the unstressed control cultures had risen to pH 8.8, conceivable due to amino acid catabolism resulting in the production of ammonia. The pH of the HCl-shocked cultures was pH 8.5, that of the HL-shocked cultures was pH 7.2, and that of the HAc/HCl-shocked cultures was pH 7.1. The pH of the HAc-shocked cultures had not changed and remained at pH 5.5 (data not shown).

3.2. Effect of acid shock on ATP levels

The initial physiological responses of *B. cereus* upon exposure to the different conditions used were studied in more detail by determining the viability through plate counts and the cellular energy status through ATP measurements (Fig. 2). Samples were taken directly before and 10, 30, and 60 minutes after exposure. In the presence of 2 mM undissociated HL or HAc/HCl, viable counts remained constant in 60 minutes of exposure, confirming the bacteriostatic conditions observed in Fig 1. In contrast, in the presence of HCl viable counts were significantly higher ($P<0.02$), indicating growth. Finally, in the presence of 15 mM undissociated organic acid the viable counts were significantly lower ($P<0.006$), indicating bactericidal conditions. To determine the effect of the different stress conditions on the energy status of the cells, ATP measurements were performed (Fig. 2B). The HCl-exposed cultures that were continuing growth showed an increase of ATP levels after 30 min. The ATP levels in the HL and HAc/HCl exposed cultures remained constant and exposure to 15 mM undissociated HAc resulted in a significant decrease in ATP levels ($P<0.005$).

Based on these results we conclude that only a pH 5.5 acid shock in the presence of 15 mM undissociated HAc caused depletion of ATP with concomitant loss of viability.

3.3 Microarray analysis

3.3.1 Effect of acid shock on gene expression

To study the impact of the different acid shock treatments on gene expression, samples taken after 10, 30, and 60 min exposure were compared to a reference sample taken immediately before acid exposure (GEO accession number GSE15140). The datasets of gene expression relative to the reference condition were used to construct a dendrogram (Fig. 3) showing differences in time and between treatments. The bactericidal HAc shock grouped apart from the other treatments that did not affect viability. From the three remaining conditions, the HAc/HCl shock and the HCl shock grouped closest together. This was unexpected, as it did not correlate with physiological responses for which the 2 mM undissociated organic acid treatments responded similar and differed from the HCl shock. This overlapping response of the HAc/HCl and HCl treatments may be due to the increase of Cl^- ions (2.5 mM and 2.8 mM, respectively) in both conditions due to the addition of HCl as the (co)acidulant. Hierarchical clustering of all genes expressed upon all acid shocks revealed groups of genes with similar expression profiles (Fig. 4) (results presented in detail below). Furthermore, it showed that the ratios obtained from samples exposed for 30 minutes represented the majority of processes that were affected at earlier and later stages, i.e., 10 and 60 minutes of exposure, respectively. Therefore, ratios obtained from samples exposed for 30 minutes were chosen for a more detailed analysis of the effects on gene expression levels.

220

221 *3.3.2 Gene expression associated with acid shock: the general acid shock response*

222 The effects of acid shock were determined by selecting all genes having
223 significant differential expression in all acid exposures or in non-lethal exposures
224 specifically (see *Supplementary material*). Genes differentially expressed in non-
225 lethal conditions were determined by selecting the genes showing significant
226 differential expression in HCl, HL, and HAc/HCl and not showing a similar
227 significant differential expression in HAc. A set of 25 genes (17 up and 8 down) was
228 differentially expressed in all acid shock conditions and a set of 146 genes (86 up and
229 60 down) was differentially expressed in all non-lethal acid shock conditions. The
230 largest impact was shown on the expression of genes involved in energy metabolism,
231 oxidative and general stress response (up-regulated) and nucleotide metabolism and
232 cell-wall biogenesis (down-regulated) (Fig. 5). Energy metabolism was mainly
233 affected in pyruvate metabolism and TCA cycle (Fig. 6). Genes involved in
234 nucleotide transport and metabolism were down-regulated upon all acid shocks, which
235 is in line with the observed inhibition of growth (Fig. 1). Cell envelope biogenesis
236 was affected in teichoic acid and capsular polysaccharide synthesis genes. Notably,
237 expression of the F_1F_0-ATPase was down-regulated in non-lethal acid shocks. Based
238 on observations that acid stress conditions induced, next to a general stress response
239 involving σ^B and ClpBC, an oxidative stress response involving superoxide
240 dismutase, catalase and iron homeostasis proteins, we conclude that reactive oxygen
241 species may be formed. At the same time the pyruvate metabolism is changing
242 dramatically, including induction of the TCA cycle and concurrent induction of
243 fermentation pathways. This may be required for maintaining intracellular ATP levels
244 (Fig. 2B) and/or the redox balance.

3.3.3 Gene expression associated with bactericidal conditions

Genes associated with bactericidal conditions were defined as differentially expressed genes in cells exposed to 15 mM undissociated HAc having different expression upon exposure to 2 mM undissociated organic acids and HCl. 137 genes (60 up-regulated and 77 down-regulated) could be associated with the bactericidal condition (see *Supplementary material*). The largest impact was on expression of genes involved in energy metabolism and electron transport (up-regulated) and on genes of unknown function (down-regulated) (Fig. 5; Fig. 6). Different fermentation pathways were induced via up-regulation of genes encoding L-lactate dehydrogenases, lactate permease, and alcohol dehydrogenases. Expression of electron transport genes was also up-regulated upon lethal HAc stress. It is conceivable that the concerted activity of NAD(P)-dependent dehydrogenases and the electron transport system is used as ultimate response to pump protons out of the cell or to restore the redox balance. Other cellular processes that were specifically affected by lethal HAc stress were transport mechanisms and cell membrane biogenesis.

3.3.4 Gene expression associated with bacteriostatic conditions

Genes associated with bacteriostatic conditions, i.e., the presence of 2 mM undissociated organic acids, were defined as all similarly differentially expressed genes (all at least two-fold up or all at least two-fold down) of the HL and HAc/HCl exposed cells having different expression from the HCl and HAc exposed cells. Our data set had 224 genes (82 up-regulated & 142 down-regulated) that were differentially expressed in the 2 mM undissociated organic acid exposures (see *Supplementary material*). The largest impact was on expression of genes involved in

oxidative stress and redox balancing (up-regulated), and amino acid transport and metabolism and membrane and cell envelope biogenesis (down-regulated). In summary, gene expression associated with bacteriostatic organic acid stress conditions differs from growth-inhibiting inorganic acid stress conditions and lethal HAc exposure by an extended oxidative stress response, which includes the expression of an additional catalase, which is conceivably required to counteract increased oxidative damage. This response, together with changes in amino acid and oligopeptide uptake and metabolism and down-regulation of lipid metabolism seems to allow for survival and maintenance of the energy status, while cell growth was inhibited.

3.3.5 Gene expression specific for HL exposure

Genes associated with HL response were defined as all differentially expressed genes of HL exposure having different expression in the HAc/HCl, HAc, and HCl exposure. Our data set had 196 genes (55 up-regulated and 141 down-regulated) that were differentially expressed upon HL exposure (see *Supplementary material*). The largest impact was on expression of genes involved in amino acid metabolism, most notably the arginine pathway, transport mechanisms and genes of unknown function (up-regulated). Several genes involved in glycolysis were also moderately up-regulated. In cell envelope biogenesis there was an up-regulation of the murein hydrolase exporter and regulator genes, but several other genes involved in cell envelope biogenesis were down-regulated. Other differentially expressed genes were mainly putative transcription regulators, ABC transporters with unknown substrate or with an unknown function.

3.3.6 Gene expression associated with non-lethal HAc exposure

Genes associated with 2 mM undissociated HAc response were defined as all differentially expressed genes of the HAc/HCl dataset having different expression in the HCl data set. This data set had 1430 genes (696 up-regulated and 734 down-regulated) that were differentially expressed upon HAc/HCl exposure (see *Supplementary materials*) compared to exposure to the same pH acidified with only HCl, indicating a tremendous impact of HAc on gene expression. The largest impact was on expression of genes involved in oligopeptide and amino acid transport and metabolism, but other pathways, most notably in redox balancing and lipid and energy metabolism, were also affected. The exposure to HAc/HCl also induced the expression of GroES and GroEL genes. There was a clear shift in carbohydrate transport and metabolism with genes encoding sugar transporters for fructose, glucose, lichenan, and trehalose being down-regulated while another glucose transport gene (BC5320) was up-regulated. In electron transport induction of expression of nitrate reductase and molybdopterin biosynthesis genes indicated a switch to anaerobic respiration under aerobic conditions and/or a rearrangement in nitrogen metabolism. For nucleotide transport and metabolism there were various changes in gene expression for purine, and pyrimidine metabolism (see *Supplementary material*). Translation, ribosomal structure and biogenesis was affected as shown by down-regulation of ribosomal protein gene expression, which is opposite from the response to HL exposure where these genes were moderately up-regulated. Also the expression of several ABC transporters with unknown substrate specificities was opposite from the response to HL exposure.

4. Discussion

In this study, *B. cereus* ATCC 14579 was exposed to pH 5.5 set with different acidulants. Depending on the acidulant used, there was a clear difference in the response, with HCl diminishing growth, 2 mM undissociated HL or HAc providing bacteriostatic conditions, that were overcome with prolonged incubation, and with 15 mM undissociated HAc, providing bactericidal conditions. The fact that organic acids display bacteriostatic or bactericidal effects at a given pH, whereas inorganic acids do not, is well-recorded and this has mainly been attributed to a less efficient lowering of the pH_i in the latter case (Brul and Coote, 1999; Cotter and Hill, 2003). However, the specifically induced transcriptional responses to the different acidulants are still unresolved and this topic will be discussed below based on our results obtained with aerobically grown *B. cereus* ATCC 14579.

The phenotypic and transcriptome responses were studied in more detail for the first 60 min of exposure. Differences in viable counts between the growth-inhibited, bacteriostatic and bactericidal conditions were observed. ATP measurements showed that the energy status is maintained within the cells for the non-lethal acid stress conditions, as ATP levels remained constant or were elevated (Fig. 2B). This may be associated with a modulation of oxidative stress response and pyruvate metabolism (Fig. 5, Fig. 6). Under bacteriostatic conditions a more stringent oxidative stress response is observed and additional modulation of amino acid and oligopeptide transport compared to conditions that diminished growth. Exposure to 15 mM undissociated HAc resulted in inactivation of cells after prolonged exposure and ATP depletion within the first hour. Next to modulation of pyruvate metabolism and oxidative stress response this may be associated with induction of alternative electron

transport systems and fatty acid biosynthesis genes. Under non-lethal acid stress conditions, rerouting of the pyruvate metabolism was indicated by induction of a butanediol fermentation pathway and part of the TCA cycle, whereas under bactericidal acid stress conditions lactate, formate, and ethanol fermentation pathways were induced. Apparently, the metabolic rerouting under bactericidal conditions is insufficient to maintain the redox balance and to generate enough ATP.

A prominent aspect of the common acid stress response is the oxidative response. This response is most pronounced for the bacteriostatic conditions, and least pronounced for the bactericidal condition (Fig. 5). This indicates that acid shock of aerobic *B. cereus* ATCC 14579 cells to pH 5.5 may cause increased formation of reactive oxygen species that need to be counteracted as reflected in the increased expression of superoxide dismutase, catalases, and nitric oxide dioxygenase. Induction of superoxide dismutase by *B. cereus* upon acid stress has been reported before (Browne and Dowds, 2002) and an oxidative stress response to mild acid stress has also been reported for *Bacillus subtilis* (Wilks *et al.*, 2009). Notably, the formation of reactive oxygen species and a role for oxidative damage in the bactericidal activity of antibiotics in *Escherichia coli* was recently established (Kohanski *et al.*, 2007). In our work, the induction of the alternative electron-transport chains and NADH-dehydrogenase indicate that the electron transport chain is affected under bactericidal conditions. Similar to the proposed bactericidal mechanism of antibiotics in *E. coli* (Kohanski *et al.*, 2007), induction of oxidative stress related genes may be a response to the formation of reactive oxygen species generated by a perturbation of the electron transport chain. The role of reactive oxygen species in the response of *B. cereus* to acid stress remains to be elucidated and is the subject of further study in our laboratory. The oxidative response in *B. cereus* appears to include

370 the formation of nitric oxide (NO) since genes encoding NO-metabolising enzymes
371 are activated. NO can be produced by nitric oxide synthase (bNOS) and this enzyme
372 has been reported to act as a fast-response protection mechanism in *B. subtilis* and
373 *Bacillus anthracis*. NO, formed from arginine, protects cells from H_2O_2-induced DNA
374 damage by inhibition of the Fenton reaction and activation of catalase (Gusarov and
375 Nudler, 2005; Shatalin *et al.*, 2008). Although bNOS is not differentially expressed,
376 which may be explained by regulation of its function on protein level (Shatalin *et al.*,
377 2008), orthologs of flavodoxins that support catalysis in *B. subtilis* (e.g., YkuN) are
378 induced under bacteriostatic organic acid stress conditions. These may be responsible
379 for enhanced production of NO from arginine. In addition, Hochgrafe and colleagues
380 (2008) proposed that NO can also protect proteins from irreversible thiol oxidation in
381 *B. subtilis* and *Staphylococcus aureus*. Our data suggest that also in *B. cereus* the
382 endogenous production of NO may provide protection against acid stress.
383 The bactericidal condition showed increased expression of lactate dehydrogenase
384 (*ldh*) and cytochrome *bd* oxidase (*cydAB*) genes. In *B. subtilis* these genes are co-
385 ordinately expressed together with the lactate permease gene *lctP* and formate-nitrite
386 transporter gene *ywcJ* and under control of the negative regulator YdiH (Rex)
387 (Larsson *et al.*, 2005). Lactate dehydrogenase, which converts lactate to pyruvate, in
388 concert with the cytochrome *bd* oxidase has been proposed to function as an
389 alternative electron transport chain (Chai et al., 2009), which may associate with the
390 oxidative response described above and may additionally contribute to the removal of
391 lactate. Together with the *alsSD* genes, *cydAB*, *ldh*, and *lctP* form a distinct regulon,
392 which is part of the larger Fnr regulon (Reents *et al.*, 2006). The *alsSD* genes encode
393 for enzymes producing acetoin from pyruvate that are involved in anaerobic
394 metabolism (Nakano et al., 1997; Fuchs et al., 2007). This metabolic shift may also

result in lower levels of lactate produced from pyruvate. The *B. subitilis alsSD* genes are strongly induced under mild acid stress conditions (Wilks *et al.*, 2009) and the *alsSD* genes in *Lactobacillus plantarum* were shown to contribute to intracellular pH homeostasis (Tsau *et al.*, 1992). In our experiments induction of the *B. cereus alsSD* genes was less pronounced under bactericidal conditions than under the milder stress conditions (Fig. 6). In contrast, *cydAB*, *ldh*, and nitrite transporter gene *ywcJ* were among the highest induced genes upon 15 mM undissociated HAc exposure. In analogy with *B. subtilis*, a changing NADH/NAD^+ ratio may be associated with the induced expression of these genes.

Adjustments to the cell-envelope (including the cell wall and membrane) may provide most optimal protection of the cell integrity under the different stress conditions, as observed for *L. plantarum* exposed to acid stress conditions (Pieterse *et al.*, 2005) and for *B. subtilis* exposed to sorbic acid stress (Ter Beek *et al.*, 2008). The response under bactericidal conditions showed some resemblance with the response of *S. aureus* cells deficit of *murF* that have reduced peptidoglycan synthesis. These cells show down-regulated expression of iron uptake associated genes, induced *ldh*, lactate permease, and formate/nitrite transporter protein genes (Sobral *et al.*, 2007). *B. cereus* cell wall metabolism was mainly affected in the non-lethal acid shock conditions by modulation of teichoic acid and capsular polysaccharide biosynthesis gene expression. Genes involved in lipid biosynthesis were down-regulated under bacteriostatic conditions, which is in line with the reduced need for cell membrane synthesis of the non-growing cells. Under bactericidal conditions however, the fatty acid biosynthesis genes were up-regulated indicating active modulation of the cell membrane under these conditions.

At similar concentrations of undissociated acid, HAc and HL evoke compound-specific responses related to specific actions of the organic acid used and/or differences in the resultant intracellular pH. Expression of a large group of ribosomal protein genes was down-regulated under HAc stress, while moderately up-regulated under HL stress. Several ABC transporters of unknown function were induced under HAc stress conditions, while repressed under HL stress conditions. There was a clear difference in catabolism of branched-chain amino acids, which may result in different adaptations to the lipid composition of the membrane. HAc stress included a shift in sugar PTS systems, and an apparent switch to anaerobic respiration. HL stress included an up-regulation of glycolysis, TCA cycle and pyruvate metabolism. Rerouting of fermentation products is also observed for *L. plantarum* under lactic acid stress conditions (Pieterse *et al.*, 2005). Up-regulation of genes involved in arginine uptake and metabolism appeared to be HL stress specific. This provides a possibility that *B. cereus* uses arginine conversion to citrulline and ammonia conceivably to counteract HL-induced acidification, which may be linked to the lactic acid-specific up-regulation of the TCA cycle.

The expression of toxin genes is an important aspect of the virulence armour of *B. cereus*. Three well-known *B. cereus* toxins are haemolysin BL (*hbl*), non-haemolytic enterotoxin (*nhe*) and cytotoxin K (*cytK*) (Stenfors Arnesen et al., 2008). The *hbl* operon was not differentially expressed upon all different acid shock exposures. The expression of the *nhe* operon was repressed specifically upon exposure to HL. The *cytK* gene, on the other hand, was up-regulated upon exposure to HL and HAc (see *Supplementary material*), indicating that exposure to organic acids may trigger *B. cereus* to increase its virulent potential. However, more research is needed to assess the impact of preservation and storage conditions on the virulence potential of *B.*

cereus. The (organic)acid shock response of the *B. cereus* type strain ATCC 14579, a so-called laboratory strain, may differ from that of industrial or environmental isolates due to pro-longed cultivation in the lab (Earl et al., 2008). However, a previous study showed that the low pH resistance of numerous food and environmental *B. cereus* isolates was highly similar and that the role of additionally acquired acid resistance mechanisms, such as ureolytic activity, was limited (Mols et al., 2008). Furthermore, the effect of culture history may also have an influence on the obtained results, as shown for acid-adapted *B. cereus* (Jobin et al., 2002; Chen et al., 2009). Thus, the effect of suboptimal growth conditions and subsequent exposure to combinations of stresses, e.g., acid and low water activity or refrigeration temperatures, remains to be elucidated.

In conclusion, we have provided a detailed insight in the different physiological and genetic responses of the food-borne pathogen *B. cereus* to acid shocks set with different acidulants. Acid shock is linked to oxidative stress response and rerouting of pyruvate metabolism. Compared to growth-inhibiting stress conditions, the bacteriostatic conditions evoke a more stringent oxidative stress response involving additional factors involved in redox reactions and conversion of reactive oxygen species. The observed differences in transcriptional responses to HAc and HL exposure may be relevant for their use as food preservatives. The HL-specific response involves fewer genes than the HAc-specific response and in some cases opposite responses were noted, i.e., with genes activated in the presence of HL, whereas they were repressed in the presence of HAc. Under minimal processing conditions these differences could be important in selecting optimal combinations of preservative agents and/or processing conditions to obtain the desired preservation effect.

Acknowledgements

The authors would like to thank Menno van der Voort (Laboratory of Food Microbiology, Wageningen University, Wageningen, the Netherlands) for technical assistance with the microarray analyses and Marcel H. Zwietering (Laboratory of Food Microbiology, Wageningen University, Wageningen, the Netherlands) for critical reading of the manuscript.

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Supplementary material

Supplementary material contains supplementary microarray analyses including tables and figures and can be found as part of the online article.

Raw and processed microarray data are available for reviewers:
http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=prwvniguuykwcjy&acc=GSE15140

Figure legends

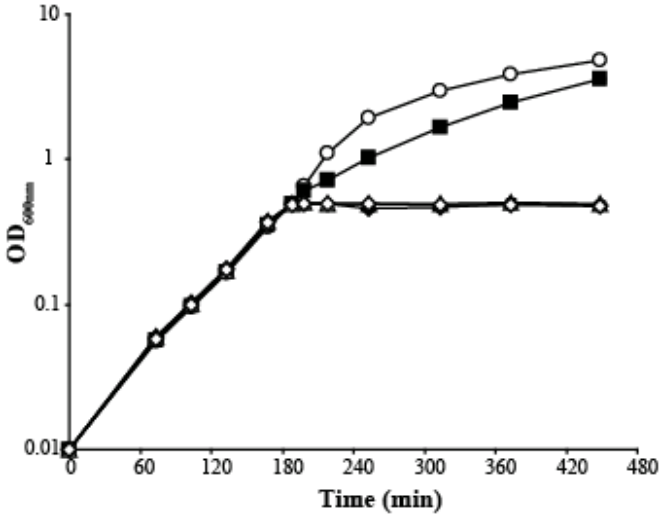
Fig. 1. Impact of acid shock on growth of *B. cereus*. Upon reaching OD 0.5, the pH of the cultures was adjusted to pH 5.5 using HCl (filled squares), HL (open triangles), HAc/HCl (filled diamonds) or HAc (open diamonds) as acidulants. The non-stressed control culture is depicted with open circles. The mean values of four cultures are shown with corresponding standard deviations indicated by error bars.

Fig. 2. Plate counts (A) and ATP measurements (B) upon acid shocks in time. Upon reaching OD 0.5, the pH of the cultures was adjusted to pH 5.5 using HCl (filled

593 squares), HL (open triangles), HAc/HCl (filled diamonds) or HAc (open diamonds) as
594 acidulants. Data are mean values from duplicate cultures and error bars indicate
595 standard deviations.
596
597 Fig. 3. Dendrogram showing clustering of up- and down-regulated genes for the
598 different acid shock conditions at time points 10, 30, and 60 minutes relative to time 0
599 just before acid shock.
600
601 Fig. 4. Hierarchical clustering of all genes expressed upon HCl (A), HL (B), HAc/HCl
602 (C) and HAc (D) treatments. Log_2 ratios of all genes at 10, 30 and 60 min of exposure
603 were clustered and displayed colorimetrically for the different acidulants used.
604 Histograms with colour scale are shown above.
605
606 Fig. 5. The response of oxidative stress, general stress response and electron transport
607 associated genes to the acid shock conditions. Bars indicate log_2 t30/t0 ratio of
608 cultures acidified with HCl (black bars), 2 mM undissociated HL (white bars), 2 mM
609 undissociated HAc (HAc/HCl, dark grey bars), and 15 mM undissociated HAc (light
610 grey bars). Oxidative stress associated genes are represented by genes encoding:
611 superoxide dismutase (*sodA*, BC5445), catalase E (*katE*, BC0863), catalase X (*katX*,
612 BC1155), nitroreductase family protein (*nfp*, BC1952), nitrite transporter (*ywcJ*,
613 BC1308), nitrite reductase (*nasD*, BC1251;), nitric oxide-dependent regulator (*dnrN*,
614 BC2137), nitric oxide dioxygenase (*hmp*, BC1448), ferrous iron transport gene (*feoB*,
615 BC0707), iron dicitrate transporter (*feuA*, BC3738), ferrichrome-transport protein
616 (*yvrC*, BC4363), ferrichrome transport gene (*fhuB*, BC4362), and dps-like protein
617 (*dps*, BC5044). Genes associated with other stresses are represented by genes

618 encoding: chaperone protein GroES (*groES*, BC0294), general stress protein 17M
619 (*yflT*, BC0998), chaperone protein DnaK (*dnaK*, BC4312), and multidrug resistance
620 protein (*yhcA*, BC4568). Electron transport related genes are depicted by genes
621 encoding: cytochrome D ubiquinol oxidase (*cydA*, BC1938), ATP synthase genes
622 *atpC* (BC5305) and *atpI* (BC5313).
623
624 Fig. 6. The responses of pyruvate metabolism genes to the acid shock conditions. Bars
625 indicate $\log_2$ t30/t0 ratio of cultures acidified with HCl (black bars), 2 mM
626 undissociated HL (white bars), 2 mM undissociated HAc (HAc/HCl, dark grey bars),
627 and 15 mM undissociated HAc (light grey bars). Dotted lines indicate 2-fold ($\log_2$ =
628 1) induction (above) or repression (below). Glycolysis genes are represented by genes
629 encoding: glyceraldehyde 3-phosphate dehydrogenase (*gapB*, BC4583),
630 phosphoglycerate mutase (*pgm*, BC5136), and enolase (*eno*, BC5135). TCA cycle
631 genes are depicted by: isocitrate dehydrogenase (*citC*, BC4593), α-ketoglutarate
632 dehydrogenase (*odhA*, BC1252), succinyl-CoA synthetase (*sucC*, BC3834). succinate
633 dehydrogenase (*sdhA*, BC4517), fumarate hydratase (*citG*, BC1712). Pyruvate can be
634 converted to lactate, butanediol formate, acetate and ethanol. The genes facilitating
635 these conversions are represented by: L-lactate dehydrogenases *ldhA* (BC4870) and
636 *ldhB* (BC4996) for lactate formation, acetolactate synthase (*alsS*, BC0883) and
637 butanediol dehydrogenase (*ydjL*, BC0668) for butanediol formation, formate
638 acetyltransferase (*pfl*, BC0491) for formate formation, and alcohol dehydrogenases
639 *adhA* (BC2220) and *adhB* (BC4365) for ethanol formation. Genes encoding acetate
640 forming enzymes were not differentially expressed and therefore acetate is shown in
641 grey. Lactate conversion forming pyruvate is depicted by 2-hydroxy-acid oxidase
642 encoding *ysfC* (BC1297).

643



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645 Fig 1

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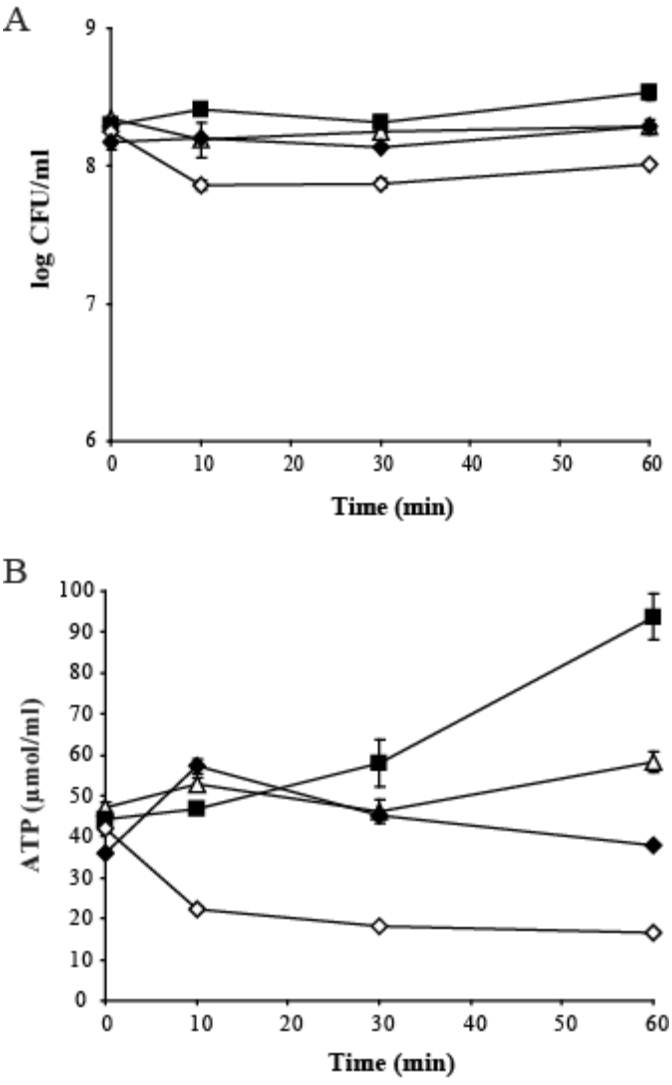

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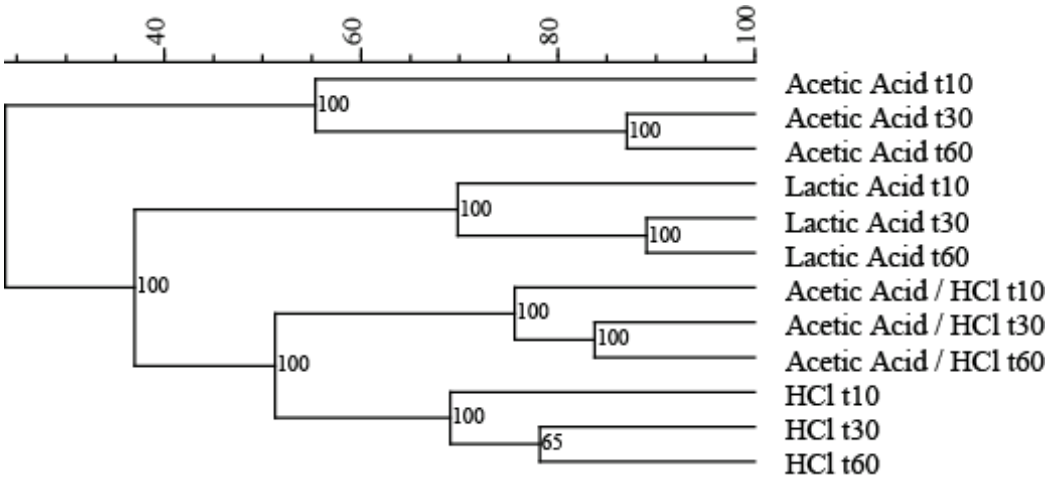
Fig 2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Acetic Acid t10
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Lactic Acid t10
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Acetic Acid / HCl t30
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HCl t10
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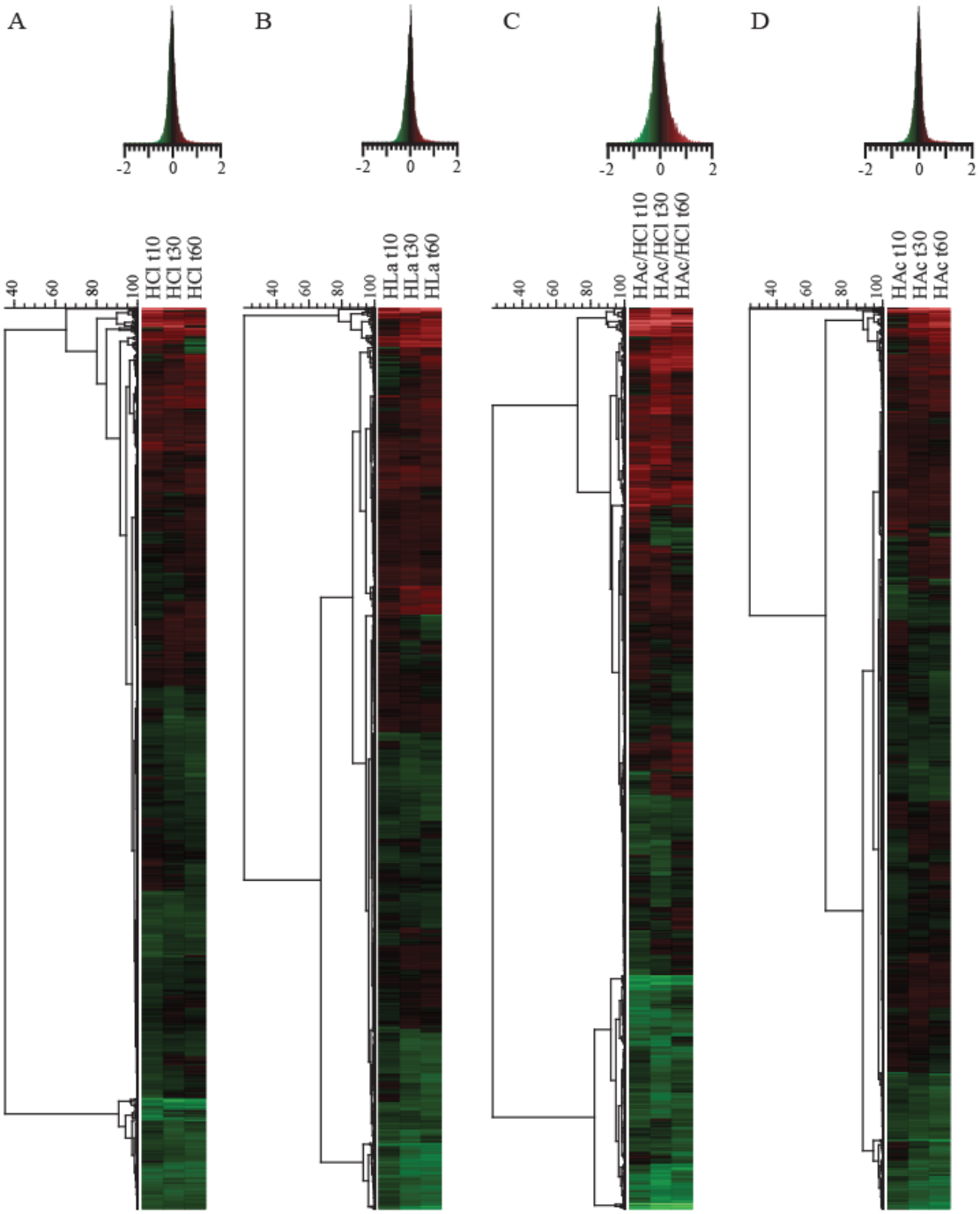
Fig 3



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654 Fig 4

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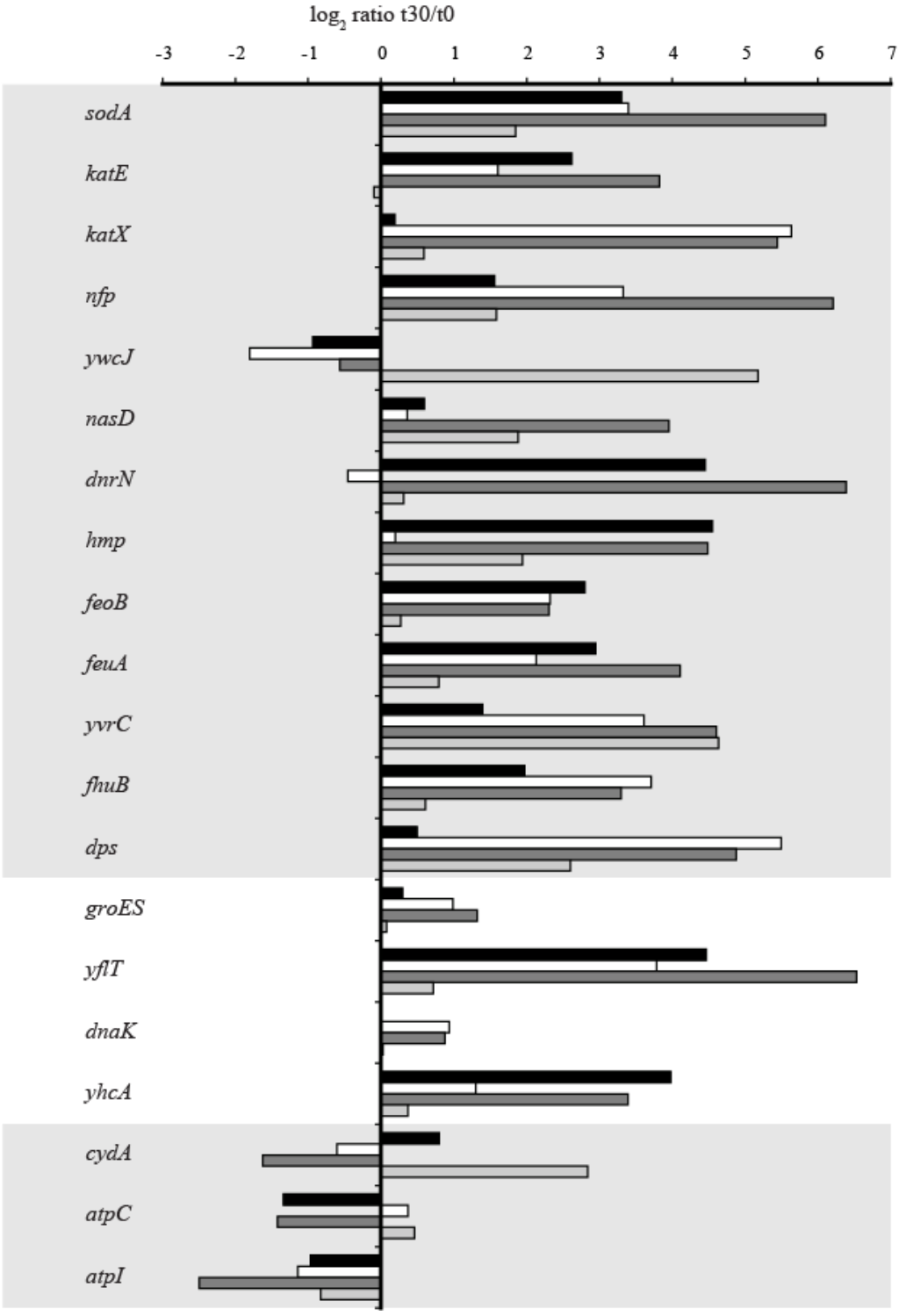

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657 Fig 5

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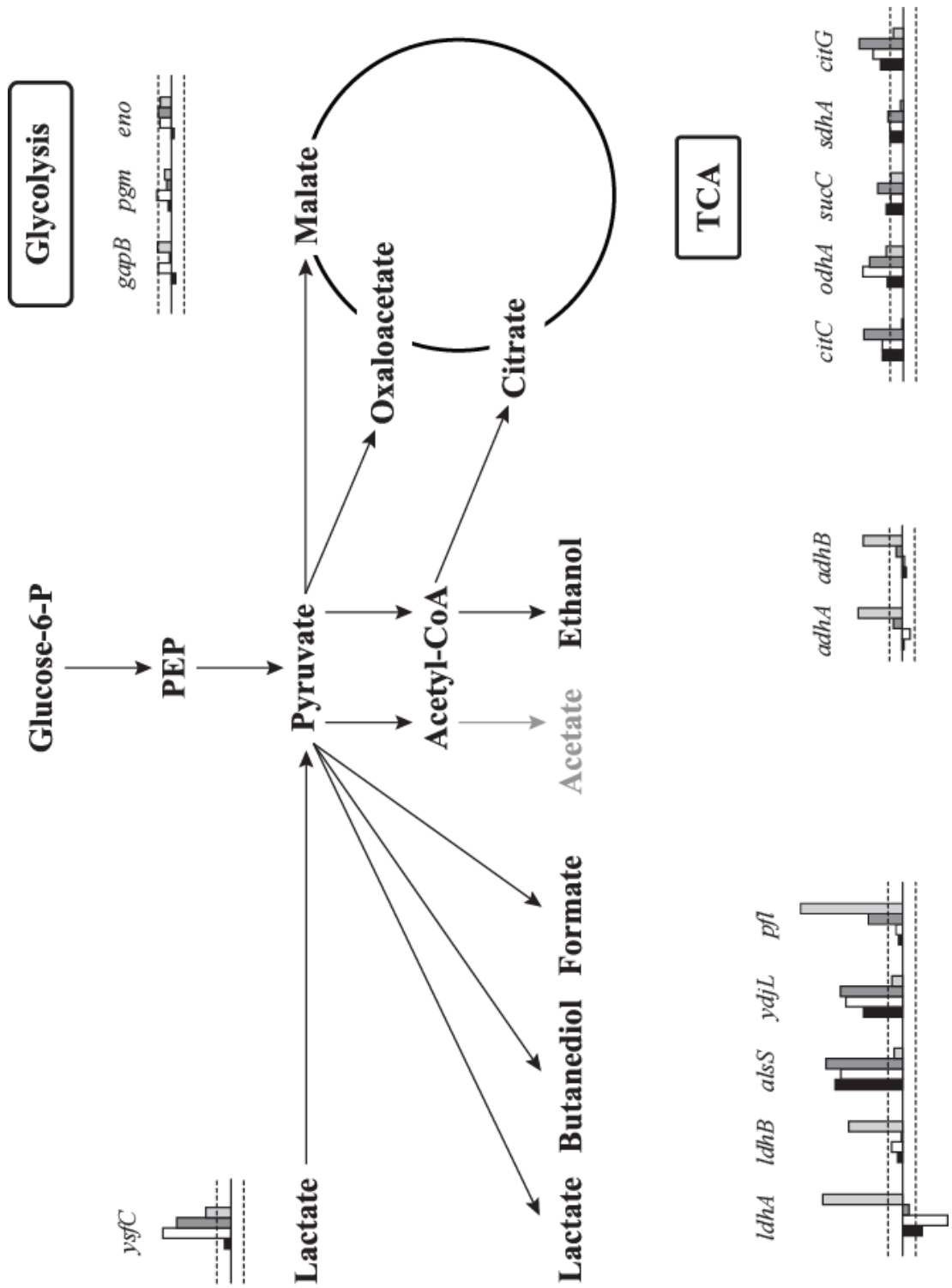
Glycolysis
gapB pgm eno
Glucose-6-P
PEP
Pyruvate
Malate
Oxaloacetate
Citrate
Acetyl-CoA
Acetate
Ethanol
Lactate
Butanediol
Formate
ysfC
Lactate
TCA
adhA adhB
citC odhA sucC sdhA citG
ldhA ldhB alsS ydjL pfl

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660 Fig 6