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Global transcription profiles and intracellular pH regulation measured in *Bacillus licheniformis* upon external pH upshifts

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Abstract For optimization of propagation conditions for an industrially used *Bacillus licheniformis*, this study examines the effect of transferring cells at the early-stationary growth phase (pH 5.3) to fresh growth medium at pH 5.0–8.0. Intracellular pH (pH_i) was measured on a single-cell level, using fluorescence ratio imaging microscopy after staining with 5(6)-carboxyfluorescein diacetate succinimidyl ester. Transcription profiles were determined using a genome DNA microarray. The optimum extracellular pH (pH_{ex}) value for growth of *B. licheniformis* was found to be pH 7.0, resulting in the shortest lag phase, highest maximum specific growth rate and maximum biomass formation. An average pH gradient ($\Delta\text{pH} = \text{pH}_i - \text{pH}_{\text{ex}}$) of approx. 1.0 was found in *B. licheniformis* 15 min after transfer to pH_{ex} 5.0–8.0. Up-regulation of genes involved in sucrose uptake at pH 7.0 could be related to the optimum growth observed. Transcription profiles indicated that the organism was experiencing phosphate starvation upon transfer to pH 7.0 and pH 8.0. Mechanisms involved in pH_i regulation appeared to include changes in fatty acid synthesis to yield a more rigid cell membrane structure at low pH_{ex} values and conversion of pyruvate to acetoin instead of acetate for neutralization of low pH_{ex} values.

Introduction

Under stressful pH conditions, regulation of intracellular pH (pH_i) in bacteria involves proton-driven transporters and more passive mechanisms, such as changes of the lipid composition of the membrane and the buffering capacity of the cytoplasm (reviewed by Hall et al. 1995). While some bacteria maintain fairly constant pH_i values upon changes in extracellular pH (pH_{ex}), i.e. pH homeostasis, others maintain a constant pH gradient ($\Delta\text{pH} = \text{pH}_i - \text{pH}_{\text{ex}}$) at least at limited pH intervals (Budde and Jakobsen 2000; Molina-Gutierrez et al. 2002; Siegumfeldt et al. 1999). For measurements of pH_i upon rapid shift of pH_{ex} , fluorescence ratio imaging microscopy (FRIM) has been used for *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Lactococcus lactis* and *Listeria innocua* stained with 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Siegumfeldt et al. 2000). The probe is membrane-permeable and is cleaved in the cytoplasm by esterases, resulting in accumulation of the fluorescent form which is intracellularly conjugated by the succinimidyl groups (Breeuwer et al. 1996). The sensitivity of the probe has been determined in *Listeria*, lactic acid bacteria, *Bacillus licheniformis* and in yeast and shown to be greatest in the range pH 5–9 with an accuracy of 0.2 pH units (Budde and Jakobsen 2000; Siegumfeldt et al. 1999, 2000; Hornbæk et al. 2002; Halm et al. 2004). Good correlations have been found in pH_i measurements made with the use of FRIM after staining with CFDA-SE and the use of ratiometric green fluorescent protein in *Lactococcus lactis* (Olsen et al. 2002). FRIM has been used for estimation of cell vitality in CFDA-SE-stained *B. licheniformis* (Hornbæk et al. 2002) and in this study, the method was applied to obtain a more thorough understanding of pH_i regulation in *B. licheniformis* upon sudden pH upshifts.

Transcriptional analysis provides a unique way of exploring the immediate genome-wide response elicited by various physical and chemical stresses as experienced

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for inocula of industrially used production strains during propagation. *Bacillus* spp such as *B. subtilis* and *B. licheniformis* are widely used industrial strains and genome-wide transcriptional profiles of *B. subtilis* have been examined upon temperature shock (Helmann et al. 2001) and alkaline stress (Wiegert et al. 2001). Transient induction of genes has been shown to reach maximum levels only 15 min after alkaline challenge (Atalla and Schumann 2003; Wiegert et al. 2001). In exponentially growing *B. subtilis*, a large subset of genes belonging to the regulon of σ^W is induced, probably as a result of impaired cell wall biosynthesis rather than specific adaptation to alkaline conditions (Cao et al. 2002; Wiegert et al. 2001). The global genetic response of *B. licheniformis* has not yet been examined and in the current study we introduce a DNA microarray covering all identified ORFs of *B. licheniformis* for transcriptional analysis at pH upshifts.

This study describes the effect of transferring *B. licheniformis* cells in the early-stationary growth phase to fresh media, exposing the cells to sudden increases in pH_{ex} from pH 5.3 to pH 6.0, 7.0 or 8.0, mimicking the transfer from seed flasks to seed tanks during propagation. The effects of pH upshifts are identified by measuring subsequent growth, ΔpH and global gene transcription.

Materials and methods

Bacterial strain and growth conditions

B. licheniformis SJ4628 was obtained from Novozymes A/S (Bagsværd, Denmark) and maintained as frozen stock at -80°C in 15% glycerol. Growth medium (SSB broth), which was used both for propagation and for cultivation of *B. licheniformis*, contained 1% (w/v) Bacto Soytone (Difco, Sparks, Md., USA), 1% (w/v) sucrose (Danisco, Denmark), 0.2% (w/v) KH_2PO_4 and 0.5% (w/v) Na_2HPO_4 . SSB broth was adjusted to pH 7.4 using 4 M NaOH and sterilized at 123°C for 20 min. *B. licheniformis* was propagated in 500-ml baffled shake-flasks in 200 ml SSB broth for 12 h at 37°C with agitation (200 rpm).

pH optimum for growth

B. licheniformis, propagated as described above, was transferred to 500-ml baffled shake-flasks at a rate of approx. 10^7 colony-forming units/ml in 200 ml SSB broth aseptically adjusted to pH 5.3 (the pH value of the propagation medium upon harvest), or to pH 6.0, 7.0 or 8.0, using 2 M HCl or NaOH. The cultures were incubated at 37°C with agitation (200 rpm) and growth was measured as the optical density at 600 nm (OD_{600}) in three independent experiments for each pH value. Data obtained from growth experiments were modelled to a modified Gombertz equation (Zwietering et al. 1990)

and growth parameters (including the lag phase, maximum specific growth rate and maximum levels of $\ln[\text{OD}_{600}(t)/\text{OD}_{600}(0)]$, where t is the sampling-time and 0 is the start-time) were estimated using the Gauss–Newton algorithm in SAS ver. 8.0 and the PROC NLIN procedure (SAS Institute, Cary, N.C., USA). Estimated growth parameters were then compared by analysis of variance, using SAS ver. 8.0 and the PROC GLM procedure.

FRIM for measurement of pH_i

After propagation, *B. licheniformis* cells were harvested for staining with the fluorescent probe CFDA-SE (Molecular Probes, Eugene, Ore., USA) according to Hornbæk et al. (2002). Following staining, cells were harvested and transferred to 500-ml baffled shake-flasks containing 200 ml SSB broth aseptically adjusted to pH values in the range pH 5.0–8.0, using 2 M HCl or NaOH. Stained cells were incubated in the SSB broth of different pH values for 15 min at 37°C under agitation (200 rpm) and then kept on ice until analysis. For FRIM analysis, CFDA-SE stained cells were immobilized on a 0.45- μm pore size membrane filter (ME 25/31; Schleicher & Schuell, Dassel, Germany) mounted in a perfusion chamber filled with 50 mM sterile-filtered potassium phosphate buffer in the range pH 5.0–8.0 supplemented with glucose (10 mM). pH_i was measured in single cells of *B. licheniformis*, as described by Hornbæk et al. (2002), using an inverted epifluorescence microscope (Zeiss Axiovert 135 TV) equipped with a Zeiss Fluor 100 \times objective (numerical aperture, 1.3), a dichroic mirror (510 nm) and an emission band pass filter (515–565 nm). Images were analysed using Metafluor ver. 4.5 software (Universal Imaging Corp., West Chester, Pa., USA), determining the ratio values by dividing the fluorescence intensity at 490 nm (pH-sensitive wavelength) by the fluorescence intensity at 435 nm (pH-insensitive wavelength) for each pixel of the image. A relationship between pH_i and the ratio values was established for the range pH 5.0–9.5 and used to calculate pH_i of each cell according to Hornbæk et al. (2002).

Isolation of RNA

B. licheniformis cells were harvested for transcriptional profiling 15 min after transfer to SSB broth at different pH values. Cells were transferred to liquid nitrogen, centrifuged (10,000 g, 5 min, 4°C) and stored at -80°C . Total RNA was isolated using the Totally RNA kit (Ambion, Huntingdon, UK), according to the manufacturer's instructions, and cell lysis facilitated by the use of 0.1-mm zirconia/silica beads (Biospec Products, Okla., USA). RNA quality was checked by the integrity of the 23S and 16S rRNA bands on agarose gel.

DNA microarray fabrication

Microscope glass slides were cleaned for 2 h in a solution containing 9% (w/v) NaOH and 35% (v/v) ethanol. They were coated by soaking for 1 h in a poly-L-lysine solution containing 15% (v/v) Poly-L-lysine and 0.1× PBS, washed three times in water, dried by centrifugation (24 g, 5 min, 20°C) and stored for at least 1 week before use. A total of 4,082 unmodified oligomers in the range 48- to 52-mers, representing all identified ORFs of the *B. licheniformis* genome, were purchased from MWG Biotech (Ebersberg, Germany) and diluted in 50% (v/v) DMSO to obtain concentrations of 50 pmol/μl. The chips were printed using a Microgrid II arrayer (BioRobotics, Huntingdon, UK) followed by rehydration over steaming water for 2 s and UV-cross linking at 60 mJ in a UV-Stratalinker 1800 (Biocrest, Amsterdam, Holland). Free poly-L-lysine groups were neutralized by washing the slides for 15 min in 1-methyl-2-pyrrolidone with 1.7% (w/v) succinic anhydride and 4.3% (v/v) boric acid (pH 8.0), followed by three washes in water. Slides were blocked by washing in 1% (w/v) bovine serum albumin, 4× SSC (3 M NaCl plus 3 M sodium citrate) and 0.5% (w/v) SDS, for 45 min at 42°C followed by five washes in water and drying by centrifugation (24 g, 5 min at 20°C). Each of the 4,082 ORFs was present four times on the *B. licheniformis* genome chip.

Probe synthesis, labelling and hybridization to DNA microarray

The transcription profile of cells transferred to SSB broth at pH 5.3 (the pH value of the propagation medium upon harvest) was compared with the transcription profiles of cells transferred to SSB broth at pH 6.0, 7.0 and 8.0. For two cultures to be compared, 25 μg total extracted RNA were indirectly labelled with Cy3 or Cy5 from the CyScribe cDNA post labeling kit (Amersham Biosciences, Buckinghamshire, UK), according to the manufacturer's instructions. Then, 3× SSC, 0.3% (w/v) SDS and 0.05% (w/v) tRNA were added to the labelled cDNA samples before hybridization to the microarray chip under a supported cover slip in a humid chamber overnight at 60°C.

Scanning of DNA microarrays and data analysis

After hybridization, the supported cover slip was gently removed by placing the chip in a buffer of 2× SSC and 0.1% SDS. The chip was washed for 5 min in each of three buffers: (a) 2× SSC + 0.1% SDS, (b) 1× SSC + 0.1% SDS and (c) 0.5× SSC, then washed in water for 10 s and immediately dried by centrifugation (24 g, 5 min, 20°C). Slides were scanned in a GMS 418 scanner (Genetic Micro Systems) using excitation wavelengths of 532 nm and 635 nm to detect Cy3 and Cy5 emission.

Data analyses were carried out using ImaGene 5.0 and GeneSight 3.5 from BioDiscovery (Calif., USA) with Lowess normalization; and statistical *t*-tests were performed on a 99% confidence level. Two individual experiments were made for each pH value. For each experiment, four replicate hybridizations were made in which the incorporated dye was reversed. Homology searches to known *B. subtilis* DNA sequences and their current functional assignments were obtained from the SubtiList database (Moszer et al. 1995).

Chemicals

Chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (Valensbæk, Denmark), unless otherwise stated.

Results

Table 1 shows the estimates of the growth parameters for *B. licheniformis* cultivated at 37°C in SSB broth adjusted to pH_{ex} values of pH 5.3, 6.0, 7.0 and 8.0, using inocula propagated to the early-stationary growth phase at pH 5.3. It appeared that the lag phases were significantly longer at pH 8.0, compared with pH_{ex} 5.3, 6.0 or 7.0 and were significantly shorter at pH_{ex} 7.0 (Table 1). The maximum specific growth rates were significantly higher for cultures grown at pH_{ex} 7.0 compared with pH_{ex} 6.0 or 8.0 (Table 1). The lowest maximum specific growth rate was found at pH_{ex} 5.3 (Table 1). Finally, it was seen that the maximum level of biomass, expressed as ln[OD₆₀₀(*t*)/OD₆₀₀(0)], increased with the initial pH of the growth medium, although not significantly in the range pH 6.0–8.0 (Table 1). Thus, using a propagation medium of pH 7.0 resulted in the shortest lag phase, the fastest growth and a maximum level of biomass for inoculation of subsequent steps of propagation and the final fermentation. At the end of cultivation, pH of the media were 5.1, 5.4, 6.2 and 6.5, using initial values of pH 5.3, 6.0, 7.0 and 8.0, respectively.

The FRIM measurements of single cells 15 min after transfer to SSB broth at pH_{ex} values in the range

Table 1 Estimates of growth parameters of *B. licheniformis* SJ4628 in SSB broth adjusted to pH_{ex} values as indicated, grown at 37°C under agitation (200 rpm). For each pH_{ex} value, growth was measured in three independent shake-flasks; and estimates of average growth parameters are shown with the 95% confidence interval determined by using the Gauss–Newton algorithm in SAS ver.8.0 and the PROC NLIN procedure. Estimates with a different number of asterisks are significantly different (*P* < 0.05)

pH _{ex}	Lag phase (h)	Maximum specific growth rate (x/h)	Maximum ln (OD ₆₀₀ (<i>t</i>)/OD ₆₀₀ (0))
5.3	0.81 ± 0.08**	1.06 ± 0.04*	5.29 ± 0.07*
6.0	0.80 ± 0.08**	1.21 ± 0.05**	5.63 ± 0.08**
7.0	0.67 ± 0.09*	1.42 ± 0.05***	5.65 ± 0.05***
8.0	1.13 ± 0.20***	1.23 ± 0.12**	5.87 ± 0.22**

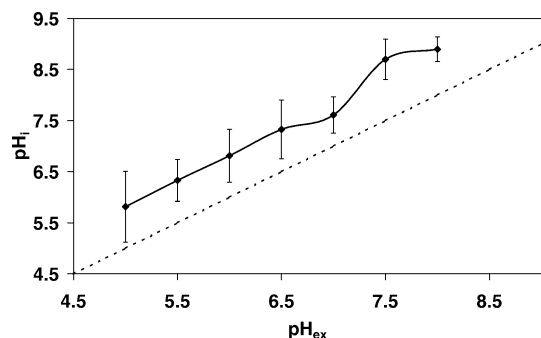


Fig. 1 Average pH_i values measured by FRIM in 60 single cells of *B. licheniformis* stained with CFDA-SE and exposed to the corresponding pH_{ex} values for 15 min. The dotted line shows pH_i = pH_{ex}.

pH 5.0–8.0 showed that *B. licheniformis* maintained an average Δ pH of approx. 1.0 pH unit (Fig. 1), independent of the pH_{ex} for the range studied.

The transcriptional changes occurring in *B. licheniformis* subjected to pH upshifts, mimicking the transfer between seed flasks and seed tanks during propagation, were revealed by a genome DNA microarray. Genes which were significantly up-regulated or down-regulated according to statistical *t*-tests performed on a 99% confidence level upon transfer from pH 5.3 to pH 5.3 (reference), or to pH 6.0, 7.0 and 8.0 are shown in Table 2. Their homology to *B. subtilis* genes (Moszer et al. 1995) is also shown (Table 2). Genes found to be significantly differentially expressed were induced or repressed at least 2.3-fold and, generally, a larger number of differentially expressed genes were observed by upshifts from pH 5.3 to 7.0 or 8.0 than by an upshift from pH 5.3 to 6.0 (Table 2). Besides, different genes were differentially expressed by transfer to pH 7.0, compared with transfer to pH 8.0. They are mentioned in the following according to their homology to *B. subtilis* genes of known function (Moszer et al. 1995).

Genes being differentially expressed at the transfer to pH 6.0, 7.0 or 8.0 compared with the transfer to pH 5.3 included genes involved in cell wall synthesis, as seen from Table 2. Synthesis of non-phosphate-containing teichuronic acid was up-regulated at pH_{ex} 8.0, whereas the synthesis of the phosphate-containing teichoic acid was down-regulated (Table 2). The phosphate transporters encoded by the *pst* operon (*pstS*, *pstA*, *pstC*, *pstBA*, *pstBB*) were increasingly up-regulated from pH 7.0 to pH 8.0, together with *phoA* encoding an alkaline phosphatase (Table 2). Furthermore, genes involved in fatty acid synthesis (*des*, *plsX*, *fabHA*) and in oxidative phosphorylation and membrane bioenergetics (*ndhF*) were up-regulated at pH 8.0 (Table 2). Genes involved in the uptake of sucrose (*sacA*, *sacP*) were significantly up-regulated at pH 7.0 (Table 2). Genes being differentially lower expressed at transfer to pH_{ex} 6.0, 7.0 and 8.0 also included genes involved in acetoin biosynthesis (*alsD*, *alsS*; Table 2). In addition, a large group of genes sharing homology to *B. subtilis*

genes of unknown function were significantly up- or down-regulated (Table 2).

Discussion

For optimization of propagation conditions for *B. licheniformis*, the present study shows the effect of pH upshifts, mimicking the transfer from seed flasks to seed tanks during propagation. Increasing pH_{ex} from pH 5.3 to pH 8.0 caused a transient growth arrest (Table 1), which has also been reported for *B. subtilis* after an alkaline shock from pH_{ex} 6.3 to pH_{ex} 8.8 (Wiegert et al. 2001). Transferring *B. licheniformis* to an initial pH_{ex} 5.3 resulted in a lower biomass (Table 1), probably due to acidification of the medium with pH_{ex} lowered to pH 5.1 after 6 h growth (data not shown). Lowering of pH_{ex} during growth has also been found for *B. subtilis* after a sudden increase in pH_{ex} from pH 6.3 to pH 8.8 or 9.0 (Wiegert et al. 2001). For seed flask inoculum propagated as described in this study, the growth experiments showed the advantage of using an initial pH_{ex} of pH 7.0 for subsequent seed tank propagation. Furthermore, DNA microarray results showed that genes associated with sucrose uptake (*sacA*, *sacP*) were up-regulated only 15 min after transfer to pH_{ex} 7.0 (Table 2), indicating an essential role of this function for initiation of growth.

Fifteen minutes after transfer to pH_{ex} values in the range pH 5.0–8.0, *B. licheniformis* maintained an average Δ pH of approx. 1.0 pH unit. For *B. subtilis*, Δ pH values at pH_{ex} 5.0 and 8.0 have been reported to be approx. 2 and 0 pH units (Breeuwer et al. 1996). However, that study was conducted on cells that had been cultured for 3 h, whereas *B. licheniformis* used for this study was cultured for only 15 min. As changes of Δ pH from 0 to approx. 2 pH units occur in *B. licheniformis* during growth (Hornbæk et al. 2002), it is difficult to compare cells in different growth phases. It was surprising to find that *B. licheniformis* adjusted the average pH_i to the high value of pH 9.0 at 15 min after transfer to pH_{ex} 8.0. However, this average pH_i of pH 9.0 may change before the initiation of growth. The growth phase of the cells possibly also affected the transcriptional profiles, as the large subset of genes controlled by σ^W previously reported to be up-regulated in exponentially growing cells of *B. subtilis* upon sudden increases in pH_{ex} from pH 6.3 to 8.8 (Wiegert et al. 2001) were not differentially transcribed in this study. Another explanation for these different findings may also be that *B. subtilis* and *B. licheniformis* induce different responses upon pH upshifts.

The DNA microarray results obtained (Table 2) strongly indicate that the cultures were experiencing a phosphate-limited situation at the high pH_{ex} values. Under phosphate-starvation conditions, there are several ways by which use of the limiting phosphate resources can be made more efficient. One way is to reduce the consumption of phosphorus for cell wall synthesis, as seen in this study at pH_{ex} 8.0 by the increased

Table 2 Transcriptional induction of *B. licheniformis* SJ4628 genes sharing homology with *B. subtilis* genes measured 15 min after transfer to pH 6.0, 7.0 and 8.0, compared with pH 5.3. Homology searches using known *B. subtilis* DNA sequences and their current functional assignments were all obtained from the SubtiList database (Moszer et al. 1995). Induction changes (*x*-fold) are listed as averages obtained from eight experiments for each comparative analysis. + and – indicate, respectively, up- and down-regulation of genes transferred to the given pH_{ex} value, compared with pH_{ex} 5.3

<i>B. subtilis</i> gene homologue	Identity (%)	Induction at pH _{ex} (<i>x</i> -fold)			Putative gene product and function
		pH 6.0	pH 7.0	pH 8.0	
Cell wall associated					
<i>tagB</i>	59.7			–2.4	Polyglycerol phosphate assembly and export, possibly involved in teichoic acid linkage unit synthesis
<i>tuaA</i>	66.0			+2.6	Synthesis of UDP-glucuronate involved in the biosynthesis of teichuronic acid
<i>tuaB</i>	66.6			+2.4	
<i>tuaC</i>	64.8			+2.9	
<i>tuaD</i>	68.9			+2.6	Synthesis of UDP-glucose 6-dehydrogenase involved in the biosynthesis of teichuronic acid
<i>ykuA</i>	64.1		–3.0	–2.3	Similar to penicillin-binding protein
<i>yrvJ</i>	70.0			+2.4	Similar to <i>N</i> -acetylmuramoyl-L-alanine amidase
Transport/binding proteins and lipoproteins					
<i>pstS</i>	73.7		+6.1	+16.5	Phosphate ABC transporter involved in high-affinity phosphate uptake
<i>pstA</i>	71.6		+3.0	+39.2	Phosphate ABC transporter (permease) involved in high-affinity phosphate uptake
<i>pstC</i>	72.4		+3.2	+29.2	
<i>PstC</i>	82.3		+3.1	+12.5	
<i>pstBA</i>	71.8		+3.8	+17.5	Phosphate ABC transporter (ATP-binding protein) involved in high-affinity phosphate uptake
<i>pstBB</i>	70.6		+3.1	+24.9	
<i>czcD</i>	58.7	–2.3	–4.9	–2.9	Cation-efflux system membrane protein, involved in potassium uptake
<i>sacA</i>	64.9		+5.3		Sucrase-6-phosphate hydrolase
<i>sacP</i>	71.6		+5.2		Phosphotransferase system (PTS) sucrose-specific enzymes IIBC component
<i>sacP</i>	75.6		+5.1		
<i>sacP</i>	55.1		+3.6		
<i>ycnJ</i>	60.0			+3.1	Similar to copper export protein
<i>ygaD</i>	57.4			+3.7	Similar to ABC transporter (ATP-binding protein)
<i>yqeW</i>	62.9		+5.2	+9.4	Similar to Na + /Pi co-transporter
<i>yurY</i>	85.4		–2.4		Similar to ABC transporter (ATP-binding protein)
<i>yvfH</i>	74.1		+2.9		Similar to L-lactate permease
<i>yvgW</i>	66.3		–2.3	–2.7	Similar to heavy metal transporting ATPase
<i>ywjA</i>	56.5			+2.8	Similar to ABC transporter (ATP-binding protein)
<i>ywoG</i>	67.0		–4.6	–4.0	Similar to antibiotic resistance protein
Metabolism of lipids					
<i>des</i>	85.4			+5.8	Fatty acid desaturase
<i>plsX</i>	73.3			+2.7	Involved in fatty acid/phospholipid synthesis
<i>fabHA</i>	79.8			+2.8	Beta-ketoacyl-acyl carrier protein synthase III, involved in fatty acid biosynthesis
<i>ydhO</i>	74.7		+2.6	+2.9	Similar to 1-acylglycerol-3-phosphate O-acyltransferase
<i>ydhO</i>	75.5		+2.6	+3.7	
<i>yhdW</i>	68.6			+4.6	Similar to glycerophosphodiester phosphodiesterase
Stress response					
<i>htrA</i>	70.4		–3.4		Serine protease (heat-shock protein)
<i>ahpC</i>	87.3		–2.4		Alkyl hydroperoxide reductase
<i>mrgA</i>	69.4		–2.5		Metalloreulation DNA-binding stress protein
<i>yvdR</i>	74.5			+2.5	Similar to molecular chaperone
Membrane bioenergetics					
<i>ndhF</i>	58.7			+4.6	NADH dehydrogenase (subunit 5)
DNA synthesis/replication					
<i>dnaA</i>	83.2		–2.8		Involved in initiation of chromosome replication
<i>dnaK</i>	77.8		–3.5	–2.5	DNA polymerase III (beta subunit)
<i>dnaH</i>	65.9			–2.3	DNA polymerase III (gamma and tau subunit)
Biosynthetic pathways					
<i>alsD</i>	68.8	–3.1	–4.8	–5.5	Alpha-acetolactate decarboxylase involved in acetoin biosynthesis
<i>alsS</i>	69.2	–4.7	–7.5	–2.9	Alpha-acetolactate synthase involved in acetoin biosynthesis
<i>citA</i>	69.5			+3.1	Citrate synthase
<i>phoA</i>	62.2			+2.8	Alkaline phosphatase
<i>pyrAB</i>			–2.9		Carbamoyl-phosphate synthetase, involved in pyrimidine synthesis
<i>pyrC</i>	73.7			–2.3	Dihydroorotase, involved in pyrimidine synthesis
<i>pyrD</i>	77.1		–2.5	–3.2	

Table 2 (Contd.)

<i>B. subtilis</i> gene homologue	Identity (%)	Induction at pH _{ex} (x-fold)			Putative gene product and function
		pH 6.0	pH 7.0	pH 8.0	
<i>pyre</i>	69.8		-2.6	-2.9	Orotate phosphoribosyltransferase, involved in pyrimidine synthesis
<i>pyrF</i>	70.1		-3.2	-3.2	Orotidine 5'-phosphate decarboxylase, involved in pyrimidine synthesis
<i>pyrK</i>	69.8		-2.4	-2.8	Dihydro-orotate dehydrogenase, involved in pyrimidine synthesis
<i>pyrP</i>	75.2			-3.2	Uracil permease, involved in pyrimidine synthesis
<i>ybcF</i>	58.4			+5.8	Similar to carbonic anhydrase
<i>yrhH</i>	58.7		-2.6	-2.3	Similar to methyltransferase
<i>YtcB</i>	69.1			+4.4	Similar to NDP-sugar epimerase
<i>YvgR</i>	75.0			+2.9	Similar to sulfite reductase
<i>YwkA</i>	74.4		-2.7	-2.7	Similar to malate dehydrogenase
Unknown genes					
<i>ybbI</i>	55.4		+2.6		Unknown
<i>ybcD</i>	63.8			+5.5	Unknown
<i>ycgM</i>	72.6		+2.6	+5.8	Unknown
<i>ydhG</i>	65.7			+4.2	Unknown
<i>ydhO</i>	74.7		+2.6	+2.9	Unknown
<i>yfhO</i>	61.5		+3.1		Unknown
<i>yfhP</i>	69.3			+2.5	Unknown
<i>yhfH</i>	70.3			-2.4	Unknown
<i>yocF</i>	70.9			+2.7	Unknown
<i>yoeB</i>	68.1		-2.7		Unknown
<i>yqjL</i>	61.1	-2.4	-3.4	-3.7	Unknown
<i>yqjM</i>	70.9		-2.8		Unknown
<i>yvgN</i>	76.9		-2.6		Unknown
<i>yvgW</i>	66.3		-2.3	-2.7	Unknown
<i>ywaE</i>	74.4			+2.8	Unknown
<i>ywbG</i>	74.0		-2.6		Unknown
<i>ywbH</i>	70.9	-2.7	-3.5	-3.3	Unknown
<i>ywkB</i>	72.0		-2.4	-3.1	Unknown
<i>yxaA</i>	63.6		+3.0		Unknown

synthesis of non-phosphate-containing teichuronic acid encoded by the *tua* operon and by the decreased synthesis of the phosphate-containing teichoic acid encoded by the *tag* operon, as previously reported for *B. subtilis* (Lahooti and Harwood 1999; Soldo et al. 1999). The expression of genes involved in cell wall anionic polymer biosynthesis is controlled by the PhoP-PhoR two-component histidine kinase response regulatory system (Liu et al. 1998; Qi and Hulett 1998a). Other members of the Pho regulon that are induced under phosphate starvation include the *pst* operon that functions as a high-affinity phosphate transport system and the *phoA* promoter encoding an alkaline phosphatase (Qi and Hulett 1998b). Upon phosphate starvation, phosphorylated PhoP (PhoP~P) binds to sites internal to the coding regions of *phoA* and *pstS* with lower concentrations of PhoP~P required for transcription of *pstS* (Qi and Hulett 1998b). In accordance with this, a high induction of the *pst* operon was observed in our study, in particular at pH 8.0 but also at pH 7.0, while *phoA* was induced to a lower extent only at pH 8.0 (Table 2).

Induction of the complete *B. subtilis pst* operon but not of any other genes of the Pho regulon has previously been identified in exponentially growing *B. subtilis* cells exposed to a sudden upshift from pH 6.3 to 8.9 (Atalla and Schumann 2003). Induction could be prevented by increasing the phosphate concentration within the growth medium; and it was concluded that increased

expression of the *pst* operon in *B. subtilis* under alkaline conditions was caused by impaired uptake of phosphate, due to interference of alkali with the three-dimensional structure of other phosphate transporters (Atalla and Schumann 2003). Increasing the pH of the growth medium provides a shift in the chemical equilibration of phosphate causing higher concentrations of PO_4^{3-} and HPO_4^{2-} and lower concentrations of H_2PO_4^- and H_3PO_4 . Calcium and magnesium supplemented from Bacto Soytone in the growth medium at concentrations of 550 ppm and 1,610 ppm, respectively, may cause the formation of poorly soluble $\text{Mg}_3(\text{PO}_4)_2$, MgHPO_4 , $\text{Ca}_3(\text{PO}_4)_2$ and CaHPO_4 at high pH values, possibly reducing the availability of phosphate. We suggest that the phosphate starvation response observed at high pH_{ex} values is connected to this precipitation of phosphate.

Passive mechanisms may be used to regulate pH_i in bacteria, such as changing the composition of the cell membrane to affect the permeability to protons, as mentioned earlier. The *fabHA* genes which are involved in the synthesis of straight-chain and branched-chain fatty acids (Choi et al. 2000) were induced in this study at pH_{ex} 8.0. This was also the case for *plsX* playing a poorly understood role in phospholipid synthesis and being located immediately upstream of the *fab* gene cluster in *B. subtilis* (Morbidoni et al. 1996). The *des* gene encoding a phospholipid desaturase was upregulated at pH 8.0 and to our knowledge has not previously

been associated with pH upshifts. Synthesis of branched-chain fatty acids and desaturation of fatty acids have previously been shown to occur in *B. subtilis* to maintain the fluidity of the membrane upon cold shocks (Aguilar et al. 1998; Klein et al. 1999). The *des* gene was detected only 15 min after cold-shock treatment of *B. subtilis* and was involved in introducing double bonds into fatty acyl chains for optimal membrane functions at temperature down-shifts (Aguilar et al. 1998; Beckering et al. 2002). Growth of *S. mutans* at pH 5.0 compared with pH 7.0 also involved a shift to higher proportions of mono-unsaturated fatty acids, assumed to be either directly involved in reduced proton permeability in acid-adapted cells or indirectly involved by influencing the F_1F_0 H^+ ATPase activity (Quivey et al. 2000). This indicates that *B. licheniformis* tries to control the permeability of the cell membrane to protons by changing the fatty acid structure corresponding to a more rigid membrane at low pH_{ex} values.

The mechanisms used by micro-organisms to survive pH stress include induction of genes encoding enzymes that help neutralize pH_{ex} , e.g. decarboxylases induced by acid pH_{ex} (as reviewed by Hall et al. 1995). In *B. subtilis*, the enzymes responsible for the formation of acetoin, acetolactate synthase and acetolactate decarboxylase, are encoded by *alsS* and *alsD*, which are transcriptionally regulated by *alsR* (Renna et al. 1993). Acetoin production, where two molecules of pyruvate condense to form acetolactate which is then converted to acetoin by decarboxylation, appeared to help *Lactobacillus plantarum* maintain pH homeostasis (Tsau et al. 1992). In *B. subtilis*, the formation of acetoin instead of acetate from pyruvate has also been suggested to prevent overacidification of the growth medium (Presecan-Siedel et al. 1999). Formation of acetoin was more pronounced at pH 6.5 than at pH 7.2 (Amanullah et al. 2001) and low pH was required for *alsS* transcriptional activation in *B. subtilis* (Turinsky et al. 2000). In this study, transcription of acetolactate decarboxylase encoded by *alsD* decreased with increasing pH_{ex} and transcription of acetolactate synthase encoded by *alsS* was also up-regulated at pH_{ex} 5.3. We suggest that the genes involved in acetoin production in *B. licheniformis* were induced at low pH_{ex} for neutralization of the growth medium by preventing the accumulation of pyruvate and the conversion of pyruvate to acetate.

In conclusion, the combination of measuring ΔpH values and the global transcriptional response during pH upshifts of *B. licheniformis* provided insight into the mechanisms playing a role in the regulation of pH_i . The results of this study suggest that pH_i regulation in *B. licheniformis* upon external pH upshifts includes changes in the composition of the cell membrane and production of metabolites neutralizing pH_{ex} . For optimization of propagation conditions for *B. licheniformis*, the initial pH of seed tank media should be adjusted to pH 7.0 to result in the early induction of genes involved in sucrose uptake and optimum growth.

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