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Genome-Wide Gene Expression Analysis of the Switch between Acidogenesis and Solventogenesis in Continuous Cultures of *Clostridium acetobutylicum*

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Key Words

Clostridium acetobutylicum · Acidogenesis · Solventogenesis · DNA microarrays · Butanol fermentation · *sol*-operon · Cellulosome

Abstract

Clostridium acetobutylicum is able to switch from acidogenic growth to solventogenic growth. We used phosphate-limited continuous cultures that established acidogenic growth at pH 5.8 and solventogenic growth at pH 4.5. These cultures allowed a detailed transcriptomic study of the switch from acidogenesis to solventogenesis that is not superimposed by sporulation and other growth phase-dependent parameters. These experiments led to new insights into the physiological role of several genes involved in solvent formation. The *adc* gene for acetone decarboxylase is upregulated well before the rest of the *sol* locus during the switch, and pyruvate decarboxylase is induced exclusively for the period of this switch. The aldehyde-alcohol dehydrogenase gene *adhE1* located in the *sol* operon is regulated antagonistically to the paralog *adhE2* that is expressed during acidogenic conditions. A similar antagonistic pattern can be seen with the two paralogs of thiolase genes, *thlA* and *thlB*. Interestingly, the genes coding for the putative cellulosome in *C. acetobutylicum* are exclusively transcribed throughout solventogenic growth. The genes for stress response are only induced during the shift but not in the course of solventogenesis when butanol is present in the culture. Finally, the data clearly indicate that solventogenesis is independent from sporulation.

Introduction

Clostridium acetobutylicum is a strict anaerobic, endospore-forming bacterium that has been used for the biotechnological production of acetone and butanol. While in the past acetone was the more valuable product, later on butanol came into focus as a bulk chemical and an excellent biofuel that can substitute products made from crude oil [Dürre, 2007, 2008; Lee et al., 2008]. Current research focuses on generating those solvents from ligno-

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cellulosic waste such as straw, as usage of this substrate does not compete with food production. Unfortunately *C. acetobutylicum* is not known to grow on cellulose despite the presence of putative cellulosome-encoding genes that have been identified in the genome [Sabathe et al., 2002]. While some of these genes have been shown to be expressed in small amounts, their function remains an unanswered question [Lopez-Contreras et al., 2003, 2004].

The classical solvent fermentation process is a batch fermentation on substrates rich in carbohydrates where *C. acetobutylicum* goes through a complete life cycle. It starts with a logarithmic growth phase producing mainly acetic and butyric acid. This type of metabolism is called acidogenesis. When the fermentation products start to accumulate and the culture is approaching the stationary phase, the metabolism of the organism undergoes a fundamental switch. The acids produced previously are taken up again and are transformed mainly to the solvents acetone and butanol thereby abating further acidification [Lee et al., 2008]. At the same time, sporulation is initiated. The production of solvents, called solventogenesis, and sporulation have been seen as somehow interconnected processes, but the nature of the signals and molecular events leading to this fundamental switch in metabolism remain unknown [Dürre et al., 2002].

Gene expression in *C. acetobutylicum* during such batch cultures has been studied by transcription analysis in considerable detail [Alsaker and Papoutsakis, 2005; Jones et al., 2008; Tomas et al., 2003]. Despite the valuable insights by those studies, many physiological processes such as differing growth rate, substrate consumption, solvent stress from product accumulation, changing pH and sporulation are intermingled with one another making it impossible to understand many details of the expression pattern. *C. acetobutylicum* can be very reproducibly grown under stable acidogenic and solventogenic conditions in phosphate-limited continuous cultures. In such cultures kept at pH 5.8 the cells show an acidogenic metabolism, while at pH 4.5 the cells grow continuously in a typical solventogenic state, producing acetone and butanol. The switch between the two states can easily be achieved in a chemostat by adjusting the pH through natural acidification [Bahl et al., 1982a, b]. This type of continuous culture was described nearly 30 years ago, but the molecular characterization is only at its beginning. Compared to batch cultures the continuous cultivation approach has the advantage that by adjustment of merely a single parameter, acidogenic growing cells can be switched to solventogenic cells while keeping the growth rate and many other parameters constant.

Results

Acidogenic and Solventogenic Continuous Cultures

During the start of the continuous culture many cells of *C. acetobutylicum* sporulate. After 115.5 h all spores were washed out and 47.5 h later the organism grows at steady-state conditions (fig. 1b; 159 h; A) in a typical butyric acid fermentation with steady-state product concentrations of 25.6 mM acetate and 51.1 mM butyrate while the pH is maintained at pH 5.8 by addition of KOH (fig. 1b). 159 h after starting the continuous culture the pH regulation was discontinued and the pH decreased in 9.5 h to pH 4.5 due to the production of butyric and acetic acid (fig. 1; between dashed lines). After the pH shift, when the culture achieved steady-state conditions while maintaining pH 4.5 (fig. 1b; R), the culture had become solventogenic and 36.8 mM butanol and 23.9 mM acetone were produced. Therefore, solely by acidification of the medium, the continuous culture switched from acidogenic to solventogenic growth. The ethanol concentration remained nearly unchanged during the entire experiment (5 mM at pH 5.8 and 7.7 mM at pH 4.5). Because the accumulation of fermentation end products needs more time in the continuous culture than in a comparable batch fermentation, the typical concentrations of solvents can be observed a few hours after the pH reached 4.5. Throughout the actual pH shift the concentration of solvents remained relatively low.

During the pH shift the glucose concentration in the medium supernatant increased from 51.5 to 91.9 mM (fig. 1a) suggesting that the glucose consumption was considerably decreased. At the same time the optical density (OD) increased and showed the highest value at pH 4.9 (fig. 1b; D). As a consequence, the maximum levels of butyrate, acetate and ethanol were also observed at this pH (fig. 1b). When the culture reached steady-state conditions at pH 4.5 the glucose concentration in the supernatant decreased to 86.4 mM.

After the OD and the glucose concentration in the medium remained stable for at least 3 volume changes, cells were collected for transcriptional analysis (fig. 1b; A and R). The OD remained constant at an OD 600 nm of 3.9 during acidogenic and solventogenic steady-state conditions (fig. 1a).

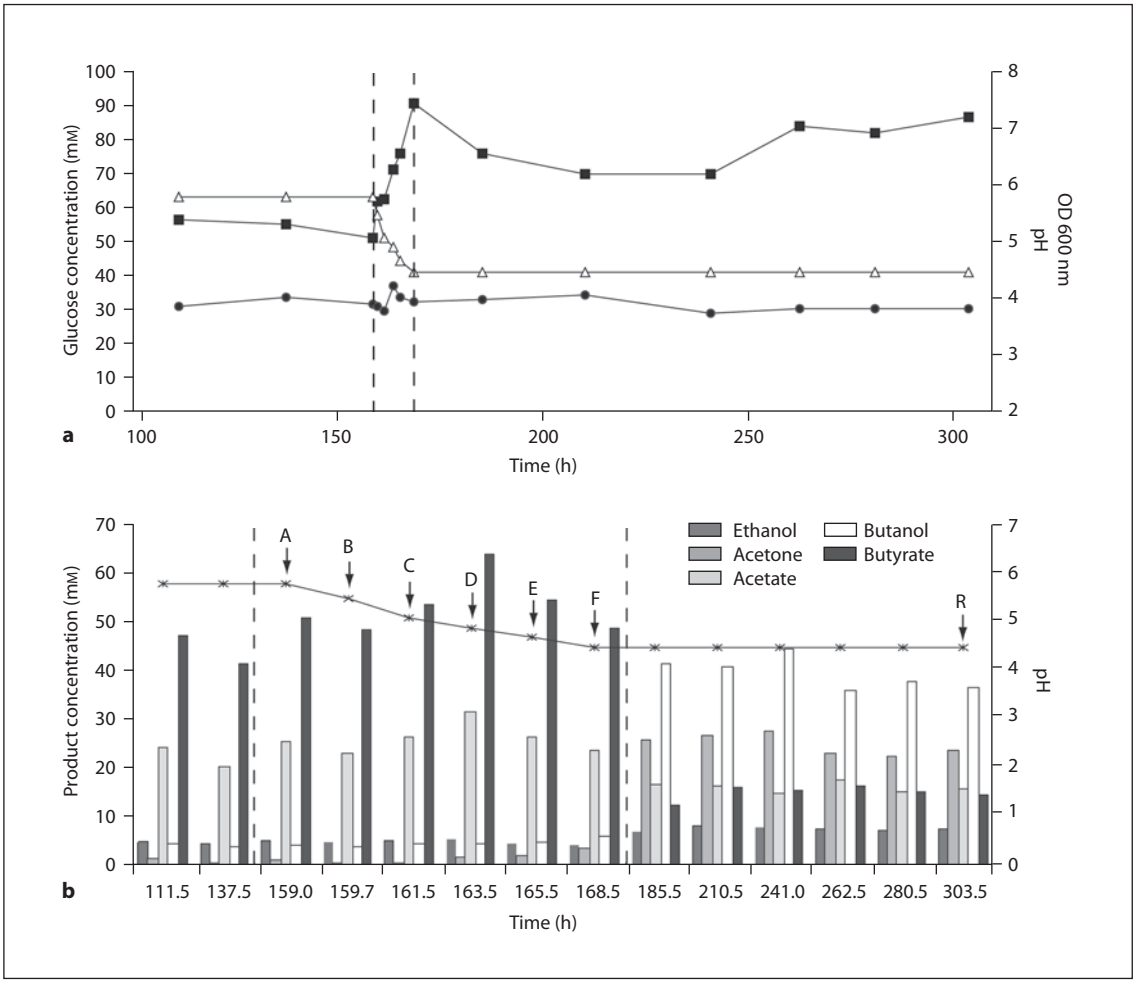


Fig. 1. Fermentation profile of a phosphate-limited continuous culture. **a** Glucose concentration in supernatant (mM) (■), OD at 600 nm (●), and pH value (△). **b** Fermentation products during the shift from acidogenesis to solventogenesis: butanol, butyrate, acetone, acetate, ethanol, and pH; dashed lines indicate dynamic pH shift. Letters represent pH values at which microarray analysis were performed. A (pH 5.8) and R (pH 4.5) represent samples taken under steady-state conditions.

Transcriptional Analysis of the Transition from the Acidogenic to the Solventogenic Growth in Phosphate-Limited Continuous Cultures

Transcriptional analysis of the transition from the acidogenic (pH 5.8) to the solventogenic (pH 4.5) growth using DNA microarrays identified 245 genes as being significantly differentially expressed. These genes were classified into four separate groups. Genes belonging to groups 1 and 2 were significantly expressed either under acidogenic or solventogenic steady-state conditions. The progression of gene expression during the transition from acidogenesis to solventogenesis is shown in figures 2 and 3. Moreover, we identified 1,849 genes that showed no significant differences in gene expressions when comparing acidogenic to solventogenic growth. However, some of these genes were induced or repressed during the transition between those steady states. Genes possessing such an expression pattern were clustered in group 3 and 4 (fig. 2, 3).

Genes Significantly Upregulated under Acidogenic Conditions

Group 1 includes 50 genes with significantly increased gene expression under acidogenic conditions at steady-state pH 5.8 as compared to solventogenic conditions (fig. 2). The expression of all genes in this group decreased

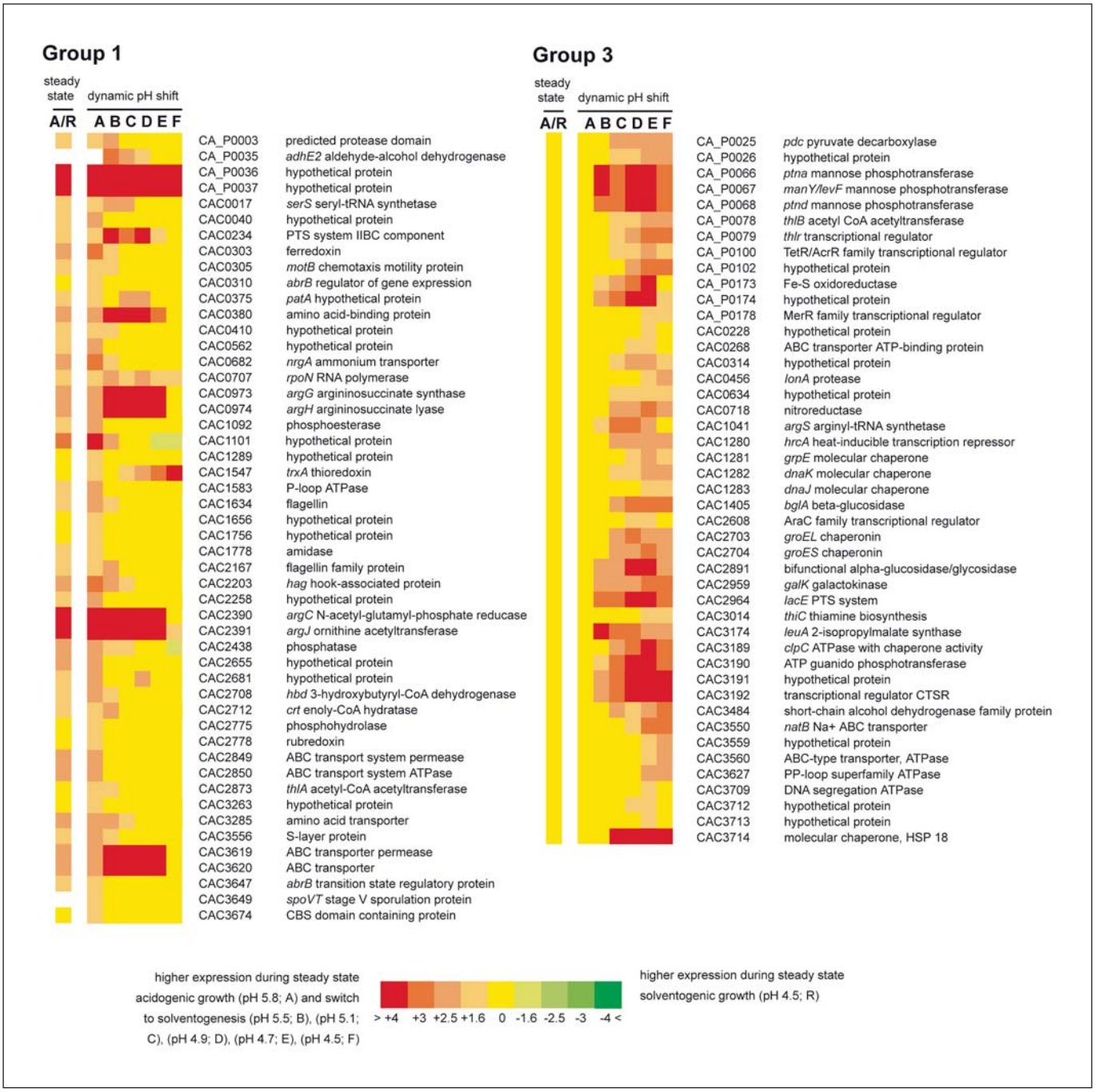
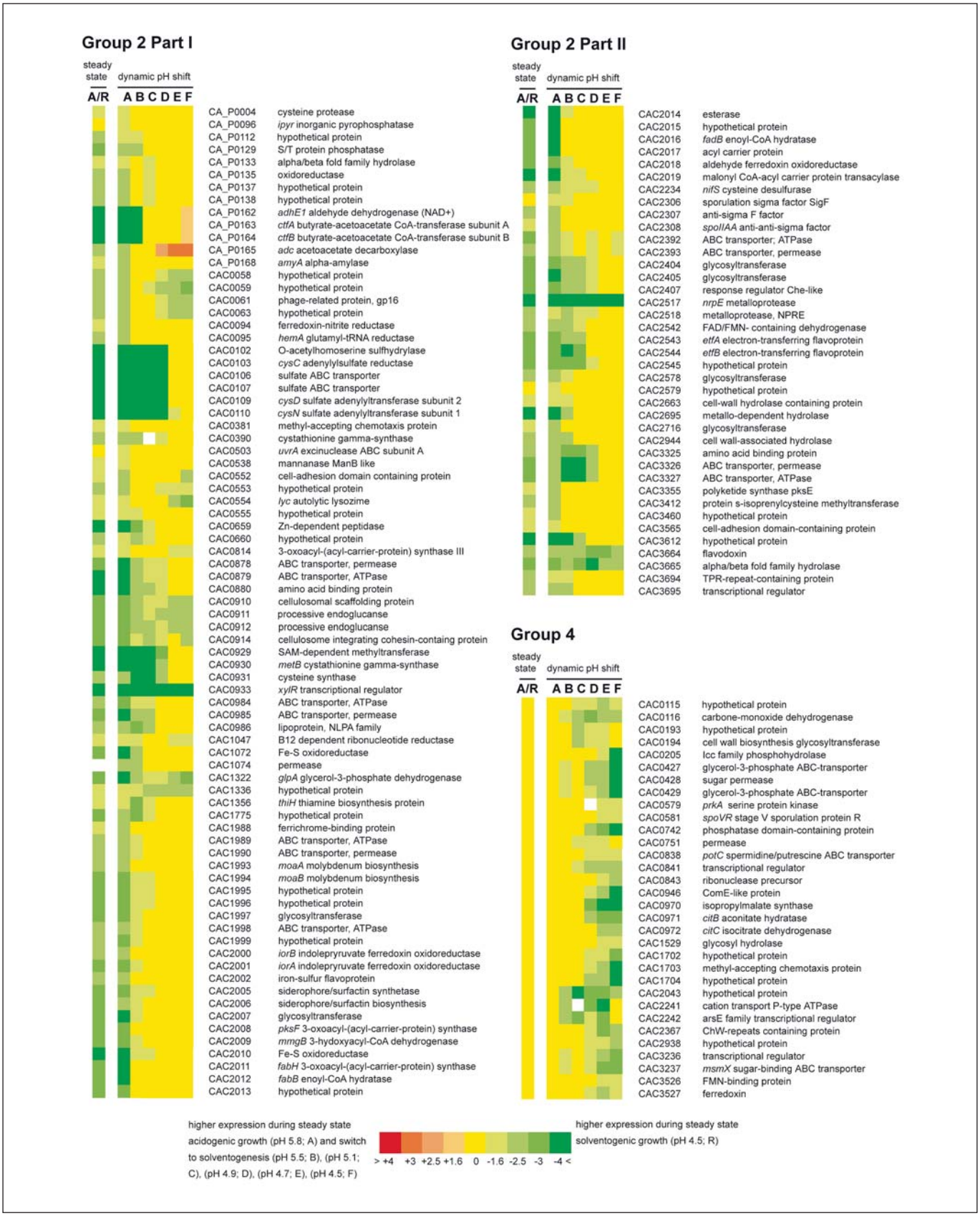


Fig. 2. Expression profiles of genes induced during acidogenic growth (group 1) and during the shift from acidogenesis to solventogenesis (group 3). RNA samples taken at the given pH values (A: pH 5.8 steady state, B–F: 5.5–4.5 dynamic shift) were hybridized against RNA from pH 4.5 steady state (R: common reference). To compare acidogenic and solventogenic steady-state conditions (A/R) the arithmetic mean of the experiment and its dye-flip was calculated. The figure shows expression ratios as logarithms to the basis of 2. White squares indicate that microarray signals were too close to the background to calculate a ratio.

Fig. 3. Expression profiles of genes induced during solventogenic growth (group 2) and genes repressed only during the shift from acidogenesis to solventogenesis (group 4). RNA samples taken at the given pH values (A: pH 5.8 steady state, B–F: 5.5–4.5 dynamic shift) were hybridized against RNA from pH 4.5 steady state (R: common reference). To compare acidogenic and solventogenic steady-state conditions (A/R) the arithmetic mean of the experiment and its dye-flip was calculated. The figure shows expression ratios as logarithms to the basis of 2. White squares indicate that microarray signals were too close to the background to calculate a ratio.


Group 2 Part I
steady state
dynamic pH shift
A/R
A B C D E F
CA_P0004 cysteine protease
CA_P0096 ipyr inorganic pyrophosphatase
CA_P0112 hypothetical protein
CA_P0129 S/T protein phosphatase
CA_P0133 alpha/beta fold family hydrolase
CA_P0135 oxidoreductase
CA_P0137 hypothetical protein
CA_P0138 hypothetical protein
CA_P0162 adhE1 aldehyde dehydrogenase (NAD+)
CA_P0163 ctfA butyrate-acetoacetate CoA-transferase subunit A
CA_P0164 ctfB butyrate-acetoacetate CoA-transferase subunit B
CA_P0165 adc acetoacetate decarboxylase
CA_P0168 amyA alpha-amylase
CAC0058 hypothetical protein
CAC0059 hypothetical protein
CAC0061 phage-related protein, gp16
CAC0063 hypothetical protein
CAC0094 ferredoxin-nitrite reductase
CAC0095 hemA glutamyl-tRNA reductase
CAC0102 O-acetylhomoserine sulfhydrylase
CAC0103 cysC adenylylsulfate reductase
CAC0106 sulfate ABC transporter
CAC0107 sulfate ABC transporter
CAC0109 cysD sulfate adenylyltransferase subunit 2
CAC0110 cysN sulfate adenylyltransferase subunit 1
CAC0381 methyl-accepting chemotaxis protein
CAC0390 cystathionine gamma-synthase
CAC0503 uvrA excinuclease ABC subunit A
CAC0538 mannanase ManB like
CAC0552 cell-adhesion domain containing protein
CAC0553 hypothetical protein
CAC0554 lyc autolytic lysozime
CAC0555 hypothetical protein
CAC0659 Zn-dependent peptidase
CAC0660 hypothetical protein
CAC0814 3-oxoacyl-(acyl-carrier-protein) synthase III
CAC0878 ABC transporter, permease
CAC0879 ABC transporter, ATPase
CAC0880 amino acid binding protein
CAC0910 cellulosomal scaffolding protein
CAC0911 processive endoglucanse
CAC0912 processive endoglucanse
CAC0914 cellulosome integrating cohesin-containg protein
CAC0929 SAM-dependent methyltransferase
CAC0930 metB cystathionine gamma-synthase
CAC0931 cysteine synthase
CAC0933 xylR transcriptional regulator
CAC0984 ABC transporter, ATPase
CAC0985 ABC transporter, permease
CAC0986 lipoprotein, NLPA family
CAC1047 B12 dependent ribonucleotide reductase
CAC1072 Fe-S oxidoreductase
CAC1074 permease
CAC1322 glpA glycerol-3-phosphate dehydrogenase
CAC1336 hypothetical protein
CAC1356 thiH thiamine biosynthesis protein
CAC1775 hypothetical protein
CAC1988 ferrichrome-binding protein
CAC1989 ABC transporter, ATPase
CAC1990 ABC transporter, permease
CAC1993 moaA molybdenum biosynthesis
CAC1994 moaB molybdenum biosynthesis
CAC1995 hypothetical protein
CAC1996 hypothetical protein
CAC1997 glycosyltransferase
CAC1998 ABC transporter, ATPase
CAC1999 hypothetical protein
CAC2000 iorB indolepyruvate ferredoxin oxidoreductase
CAC2001 iorA indolepyruvate ferredoxin oxidoreductase
CAC2002 iron-sulfur flavoprotein
CAC2005 siderophore/surfactin synthetase
CAC2006 siderophore/surfactin biosynthesis
CAC2007 glycosyltransferase
CAC2008 pksF 3-oxoacyl-(acyl-carrier-protein) synthase
CAC2009 mmgB 3-hydoxyacyl-CoA dehydrogenase
CAC2010 Fe-S oxidoreductase
CAC2011 fabH 3-oxoacyl-(acyl-carrier-protein) synthase
CAC2012 fabB enoyl-CoA hydratase
CAC2013 hypothetical protein
Group 2 Part II
steady state
dynamic pH shift
A/R
A B C D E F
CAC2014 esterase
CAC2015 hypothetical protein
CAC2016 fadB enoyl-CoA hydratase
CAC2017 acyl carrier protein
CAC2018 aldehyde ferredoxin oxidoreductase
CAC2019 malonyl CoA-acyl carrier protein transacylase
CAC2234 nifS cysteine desulfurase
CAC2306 sporulation sigma factor SigF
CAC2307 anti-sigma F factor
CAC2308 spoIIAA anti-anti-sigma factor
CAC2392 ABC transporter; ATPase
CAC2393 ABC transporter, permease
CAC2404 glycosyltransferase
CAC2405 glycosyltransferase
CAC2407 response regulator Che-like
CAC2517 nrpE metalloprotease
CAC2518 metalloprotease, NPRE
CAC2542 FAD/FMN- containing dehydrogenase
CAC2543 etfA electron-transferring flavoprotein
CAC2544 etfB electron-transferring flavoprotein
CAC2545 hypothetical protein
CAC2578 glycosyltransferase
CAC2579 hypothetical protein
CAC2663 cell-wall hydrolase containing protein
CAC2695 metallo-dependent hydrolase
CAC2716 glycosyltransferase
CAC2944 cell wall-associated hydrolase
CAC3325 amino acid binding protein
CAC3326 ABC transporter, permease
CAC3327 ABC transporter, ATPase
CAC3355 polyketide synthase pksE
CAC3412 protein s-isoprenylcysteine methyltransferase
CAC3460 hypothetical protein
CAC3565 cell-adhesion domain-containing protein
CAC3612 hypothetical protein
CAC3664 flavodoxin
CAC3665 alpha/beta fold family hydrolase
CAC3694 TPR-repeat-containing protein
CAC3695 transcriptional regulator
Group 4
steady state
dynamic pH shift
A/R
A B C D E F
CAC0115 hypothetical protein
CAC0116 carbone-monoxide dehydrogenase
CAC0193 hypothetical protein
CAC0194 cell wall biosynthesis glycosyltransferase
CAC0205 lcc family phosphohydrolase
CAC0427 glycerol-3-phosphate ABC-transporter
CAC0428 sugar permease
CAC0429 glycerol-3-phosphate ABC-transporter
CAC0579 prkA serine protein kinase
CAC0581 spoVR stage V sporulation protein R
CAC0742 phosphatase domain-containing protein
CAC0751 permease
CAC0838 potC spermidine/putrescine ABC transporter
CAC0841 transcriptional regulator
CAC0843 ribonuclease precursor
CAC0946 ComE-like protein
CAC0970 isopropylmalate synthase
CAC0971 citB aconitate hydratase
CAC0972 citC isocitrate dehydrogenase
CAC1529 glycosyl hydrolase
CAC1702 hypothetical protein
CAC1703 methyl-accepting chemotaxis protein
CAC1704 hypothetical protein
CAC2043 hypothetical protein
CAC2241 cation transport P-type ATPase
CAC2242 arsE family transcriptional regulator
CAC2367 ChW-repeats containing protein
CAC2938 hypothetical protein
CAC3236 transcriptional regulator
CAC3237 msmX sugar-binding ABC transporter
CAC3526 FMN-binding protein
CAC3527 ferredoxin
higher expression during steady state
acidogenic growth (pH 5.8; A) and switch to solventogenesis (pH 5.5; B), (pH 5.1; C), (pH 4.9; D), (pH 4.7; E), (pH 4.5; F)
> +4 +3 +2.5 +1.6 0 -1.6 -2.5 -3 -4 <
higher expression during steady state
solventogenic growth (pH 4.5; R)

3

considerably during the transition to the solventogenic phase, except the two hypothetical proteins CAP0036 and CAP0037. These genes were highly upregulated at acidogenic and transition pH values but under solventogenic steady-state conditions their gene expression was strongly repressed. Genes involved in the arginine biosynthesis (CAC0973, CAC0974, CAC2390 and CAC2391) were strongly expressed during the acidogenic phase. Moreover, an amino acid transport system (CAC3619 and CAC3620) showed the same expression pattern. Some genes encoding for flagellin family proteins (CAC1634, CAC2167 and CAC2203) were also induced during the acidogenic and repressed during transitional and solventogenic phase. These observations are in agreement with the hypothesis that expression of motility genes might be downregulated during solventogenesis [Tomas et al., 2003; Tummala et al., 2003]. In *C. acetobutylicum* ATCC 824, three putative homologs of *abrB* (CAC0310, CAC1941 and CAC3647), an inhibitor of sporulation [Benson et al., 2002; Perego et al., 1988; Strauch et al., 1989a, b; Vaughn et al., 2000], were identified [Nolling et al., 2001]. As described before [Scotcher et al., 2005], expression was not only observed for *abrB* CAC0310 but also for CAC3647. The expression of these two genes was significantly upregulated during acidogenic growth while transcription decreased during the transition to solventogenic growth (fig. 2). The genes coding for the conversion of acetyl-CoA to crotonyl-CoA *(thlA*, *hbd*, *crt)* were upregulated under acidogenic conditions and strongly reduced in expression during the transition phase and during solventogenic growth. This stands in contrast to transcriptional results from batch fermentations on CGM medium where the expression of these genes was reported to be induced during the transition from acidogenesis (exponential growth) to solventogenesis (stationary phase) [Alsaker and Papoutsakis, 2005].

The aldehyde/alcohol dehydrogenase (AADH) *adhE2* (CAP0035) was highly induced under steady-state conditions during acidogenic growth (pH 5.8). Within the pH shift to solventogenesis (pH 4.5) the gene became nearly completely repressed. The same was observed in the real-time analysis of this gene (fig. 4). The *adhE2* gene which is encoded on the pSOL1 megaplasmid has previously been described to be responsible for butanol production in cultures with a high NADH/NAD^+ ratio [Fontaine et al., 2002].

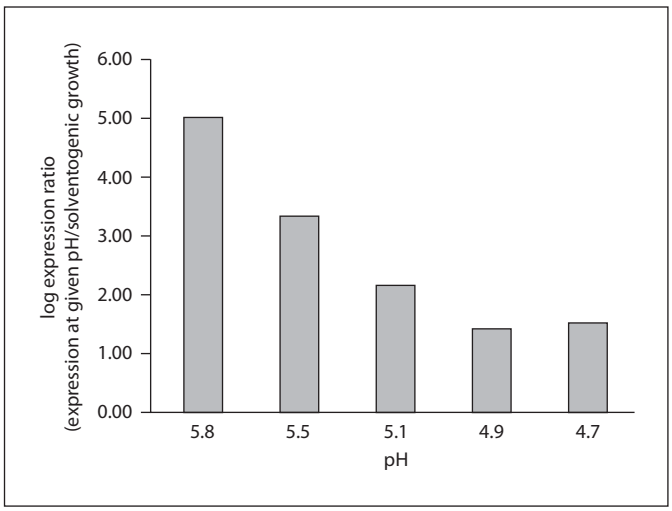


Fig. 4. Gene expression of *adhE2* (CA_P0035) during the pH shift. Verification of DNA microarray data by real-time RT-PCR. RNA from a sample at solventogenic (pH 4.5) steady-state conditions was used as a common reference.

Genes Significantly Upregulated under Solventogenic Conditions

Comparing acidogenic to solventogenic growth, 118 genes were significantly upregulated during solventogenesis. We have classified these genes as group 2 (fig. 3). Among them are some endoglucanases (CAC0910–CAC0912 and CAC0914), the transcriptional regulator *xylR* (CAC0933), the glycerol-3-phosphate dehydrogenase *glpA* (CAC1322) and a flavodoxin (CAC3664). They show nearly no changes in gene expression during acidogenic growth and the transition.

Despite the fact that *C. acetobutylicum* ATCC 824 is not known to grow on cellulose, genome sequencing identified an operon coding for a putative cellulosome (CAC0910–CAC0919) [Lopez-Contreras et al., 2003]. Overall, the functionality and role of the cluster has remained obscure. Our expression data of the cellulosome-like gene cluster demonstrate that the genes coding for cellulosomal scaffolding protein, as well as *celF* and *celA* [Lopez Contreras et al., 2003] (CAC0910–CAC0912), were highly induced during solventogenic steady-state conditions. In contrast, these genes were very weakly transcribed during the acidogenic growth or during the switch to solventogenesis. The other genes of the cluster (CAC0913–CAC0919) were also clearly induced under solventogenic conditions (fig. 6), but we did not formally include them in group 2, because we could not calculate an expression ratio due to the fact that those genes, with

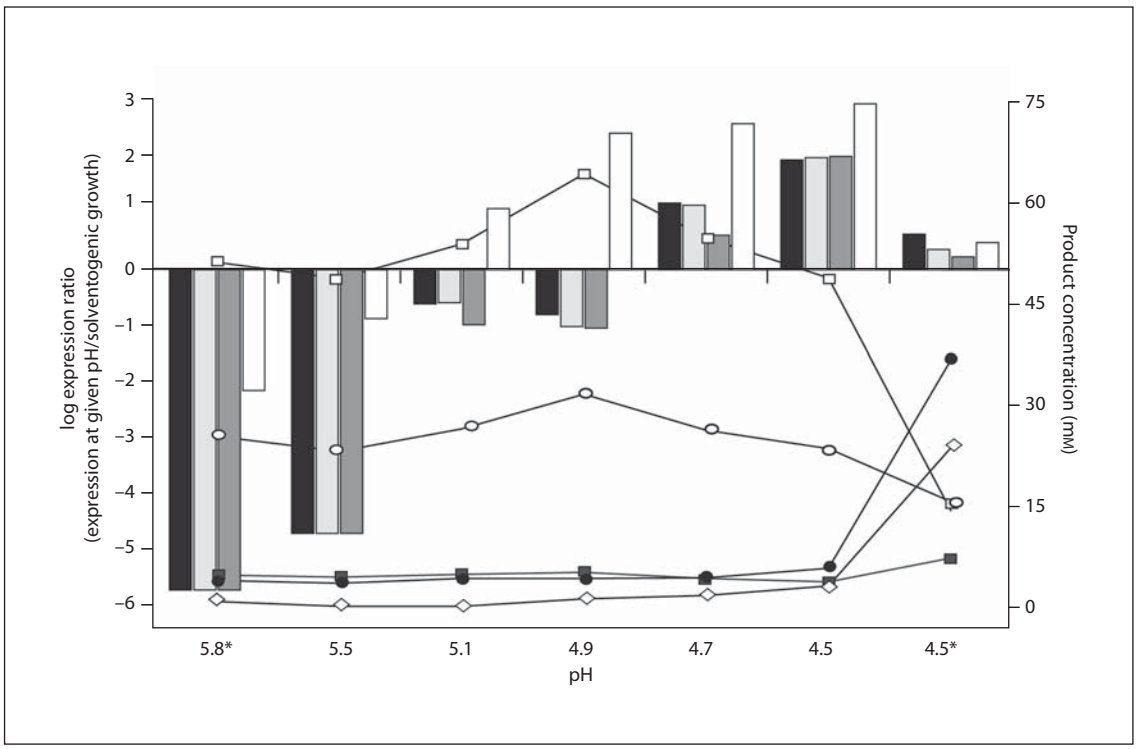


Fig. 5. Fermentation products and expression profiles of genes involved in the metabolic switch from acidogenesis to solventogenesis. Expression of *adhE1* (CAP0162) black bars, *ctfA* (CAP0163) light gray bars, *ctfB* (CAP0164) dark gray bars, *adc* (CAP0165) white bars. Fermentation products: butanol (●), acetate (○), ethanol (■), and acetone (◇). * Samples taken during steady-state conditions.

the exception of CAC0914, were completely repressed during the acidogenic growth and the switch to solventogenesis. Therefore for these conditions the signal intensity was not significantly above background. Figure 6 shows the values of signal intensity.

Other genes were also strongly induced during the metabolic switch and solventogenic growth. These include genes involved in the cysteine and sulfur metabolism (CAC0102, CAC0103, CAC0109, CAC0110, CAC0390, CAC0930 and CAC0931). The same pattern of transcriptional regulation was observed for two gene clusters encoding amino acid transport systems (CAC0878–CAC0880 and CAC3325–CAC3327).

Genes involved in fatty acid synthesis [Alsaker and Papoutsakis, 2005] were also induced during solventogenesis. These genes are located in a large gene cluster (CAC1988–CAC2019) which contains genes coding for a 3-hydroxyacyl-CoA dehydrogenase (*mmgB,* CAC2009), two *fadB* enoyl-CoA hydratases (CAC2012, CAC2016), one acyl carrier protein (ACP) (CAC2017) and malonyl CoA-ACP transacylase (CAC2019). Furthermore, there are two 3-oxoacyl-ACP synthases on the large gene cluster (CAC2008, CAC2011). All genes within this locus, for which an expression ratio has been successfully determined (CAC1988–CAC2019), were repressed throughout acidogenic growth and became induced during the shift to solventogenesis and solventogenic growth. A third paralog of a 3-oxoacyl-ACP synthase (CAC0814), which is not located in the mentioned large gene cluster, was also induced during solventogenic steady-state conditions. Transcriptional data from batch cultures showed that nearly all genes of the fatty acid synthesis cluster were strongly upregulated in the stationary phase [Alsaker and Papoutsakis, 2005]. These results are consistent with our data which also indicate a strong expression during the transition to solventogenesis and solventogenic growth (fig. 3). Genes coding for glycosyltransferases (CAC2007, CAC2404–CAC2405, CAC2578 and CAC2716) responsible for the biosynthesis of oligosaccharides were inhibited throughout the acidogenic growth and induced during the transition from acidogenesis to solventogenesis and solventogenic growth. The same was observed for

different cell wall hydrolases (CAC0554, CAC2663, CAC2695 and CAC2944) and cell-adhesion domain-containing proteins (CAC0552, CAC3565).

Several well-known solventogenic genes such as the acetoacetate decarboxylase *adc* (CAP0165) and the *sol*-operon (CAP0162–CAP0164), containing the alcohol/aldehyde dehydrogenase *adhE1* (CAP0162) and the butyrate-acetoacetate CoA-transferase *ctfA-ctfB* (CAP0163, CAP0164) [Fischer et al., 1993], became highly induced during the transition from acidogenic to solventogenic growth. While the *sol*-locus reached its highest expression level at the end of the pH shift (pH 4.5), the acetoacetate decarboxylase (*adc*) gene, transcribed from its own promoter [Gerischer and Dürre, 1992], was already significantly upregulated at pH 4.9. Moreover, in the transition phase these four genes possessed higher transcription levels than those observed at steady-state conditions at pH 4.5 (fig. 3, 5). Also included in group 2 is the tricistronic *sigF* operon (CAC2306–CAC2308) which codes for a sigma factor SigF, an anti-sigma F factor and an anti-anti-sigma factor (antagonist of SpoIIAB). The *sigF*-operon was repressed during acidogenic growth (pH 5.8) while the transcription was induced during the transition to solventogenic growth (fig. 3). The transcriptional level of *spo0A* (CAC2071) was nearly constant throughout the entire experiment.

Genes Significantly Induced Only during the Transition from Acidogenesis to Solventogenesis

Comparing steady-state conditions of acidogenic (pH 5.8) and solventogenic (pH 4.5) growth, we identified 1,849 genes that showed no significant change in expression ratio. But 45 of these genes became highly induced only during the transition from acidogenic to solventogenic growth. We classified genes with this expression pattern as group 3 (fig. 2). The pyruvate decarboxylase gene (*pdc* CAP0025) which is located on the pSOL1 megaplasmid belongs to this group. A BLAST search showed that *C. acetobutylicum* is the only *Clostridium* sequenced so far that possesses a gene coding for pyruvate decarboxylase. Moreover, the transcriptomic data of the dynamic pH shift show that a second thiolase (*thlB* CAP0078) was strongly upregulated only during the metabolic switch (points C–F). The expression ratio changes from non-regulated (yellow at pH 5.8 = A) to induced (red below pH 5.1 = C). In contrast to the Northern blot analysis published for *thlA* and *thlB* from continuous cultures of *C. acetobutylicum* [Winzer et al., 2000], a somewhat different expression pattern of the two thiolases (*thlA* CAC2873 and *thlB* CAP0078) was observed. While the chromosome-encoded thiolase *thlA* (CAC2873) was induced under acidogenic conditions (shown in group 1), the thiolase *thlB* located on the pSOL1 megaplasmid (CAP0078) was highly induced only during the transition state.

Also, genes involved in stress response of *C. acetobutylicum* belong to group 3. The expression of the *ctsR-yacH-yacI-clpC* operon (CAC3192–CAC3189) was already induced at pH 5.5, while the gene coding for the molecular chaperone Hsp18 as well as the transcripts of *hrcA-grpE-dnaK-dnaJ* (CAC1280–CAC1283) and *groEL-groES* (CAC2703–CAC2704) became upregulated when the pH values fell below pH 5.5. Furthermore, an operon which is predicted to code for a mannose uptake coupled phosphotransferase system (CAP0066–CAP0068) was highly induced only during the pH shift.

Genes Significantly Repressed Only during the Transition from Acidogenic to Solventogenic Growth but Not under Steady-State Conditions

The gene for carbon-monoxide dehydrogenase (CAC0116) is one of the genes which are only repressed during the pH shift that we classified as group 4. Moreover, a cluster of genes coding for a glycerol-3-phosphate transport system (CAC0427–CAC0429) was strongly inhibited (fig. 3).

Furthermore, while *C. acetobutylicum* is not able to use the full tricarboxylic acid cycle, the gene expression of aconitase (*citB* CAC0971) and isocitrate dehydrogenase (*citC* CAC0972) was reduced only within the pH shift. The transcription of *citBC* was significantly repressed at pH values below 4.9 and 4.7, respectively (fig. 3).

Discussion

DNA microarray analysis of a phosphate-limited continuous culture offers a unique physiological technique to study the metabolic details of acidogenesis and solventogenesis and the transition between these metabolic states in *C. acetobutylicum*. In contrast to pH-controlled batch fermentation studies, we were able to clearly separate solventogenesis from sporulation. Most important for the interpretation of microarray data was that the growth rate was kept constant, excluding genes from the analysis that were merely growth-rate- or growth-phase-dependent. Moreover, as compared to batch culture, we had no constantly changing substrate and product concentrations that influenced the transcriptome. The fermentation profile (fig. 1b) showed that the metabolic switch

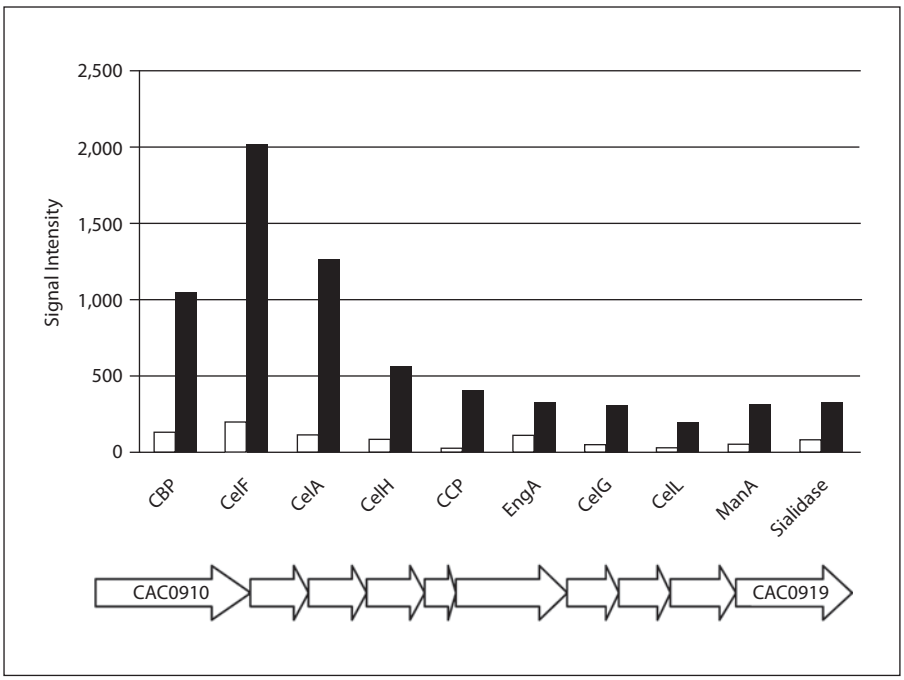


Fig. 6. Signal intensity of the cellulosome-like operon (CAC0910–CAC0919) under acidogenic and solventogenic steady-state conditions. Signal intensity at solventogenic growth (pH 4.5; R), black bars; signal intensity at acidogenic growth (pH 5.8; A), white bars.

from acidogenesis to solventogenesis was only triggered by acidification from pH 5.8 to 4.5. In contrast to former reports of continuous culture experiments [Grupe and Gottschalk, 1992] there was no significant difference of the OD when comparing acidogenic and solventogenic growth. An additional aspect of continuous cultures was that the cells were constantly dividing and therefore always had to transcribe the genes they needed to adjust their metabolism correctly. It is much more difficult to analyze solventogenesis in the stationary phase of a batch culture by transcriptomics as one has no information on the stability of enzymes formed during the acidogenic exponential growth.

During the transition from acidogenesis to solventogenesis the glucose concentration in the medium supernatant increased, suggesting that the glucose uptake rate was slowed down (fig. 1a). The different glucose consumption rates in the acidogenic and solventogenic steady-state conditions would agree with the proposal that butanol had a negative effect on the cell membrane and on glucose uptake [Bowles and Ellefson, 1985; Ounine et al., 1985]. However, these results were not consistent with a butanol-inhibited uptake or consumption during the transition phase, because there was no significant butanol concentration observed at this point of time. For this reason, we suggest that the increase of the glucose concentration in the medium supernatant reflected a profound change in carbon flow through the central metabolism. During acidogenesis all the carbon enters the metabolism via glucose. In contrast, during the shift, huge amounts of acids are taken up as additional entries of carbon in the metabolism. This has to result in a changed stoichiometry of the metabolism where less glucose is required just to provide the reduction equivalents for acid reduction. After the shift to solventogenesis, the concentration of acids in the medium has decreased and more carbon enters the central metabolism from glucose again. Under solventogenic steady-state conditions, less carbon from glucose seems to be required to maintain the same growth rate than under acidogenic steady state.

Our data also shed new light on the conditions leading to the expression of genes encoding cellulosome-like proteins and their potential role for *C. acetobutylicum*. Despite the fact that *C. acetobutylicum* is not known to grow on cellulose, genome sequencing identified a gene cluster coding for a putative cellulosome (CAC0910–CAC0919) [Lopez-Contreras et al., 2003]. This gene cluster shows high homology in gene organization and amino acid sequence to simple cellulosome systems as found in *C. cellulolyticum* [Belaich et al., 1997], *C. josui* [Kakiuchi et al., 1998], and *C. cellulovorans* [Tamaru et al., 2000]. While Western blot analysis failed to detect the putative family 9 endoglucanase CelG (CAC0916) in ex-

tracellular medium from cells grown on glucose [Lopez-Contreras et al., 2003], a unique cellulase that belongs to glycoside hydrolase family 48 CelF (CAC0911) was detected by Western blot analysis in cultures grown on Avicel treated with Celluclast [Lopez-Contreras et al., 2004]. In our hands the genes of the cellulosome-like operon (CAC0910–CAC0919; fig. 6) were strongly expressed only during solventogenic steady-state conditions but not during acidogenic growth and the metabolic switch. As glucose was present in our experiment throughout solventogenic growth, the cellulosome genes were not catabolite repressed which fits to the observation that no catabolite-repressing elements are present in this locus [Lopez-Contreras et al., 2004]. Therefore, beside the pH, the presence of solvents could be the trigger for their transcription. The rational of this is not yet clear. We propose that in natural habitats of *C. acetobutylicum*, solventogenesis is typically induced when easily fermentable carbon sources are already depleted by acidogenesis, which may drive the cells to adjust to the utilization of substrates that are harder to break down such as non-crystalline cellulose or derivatives. Simultaneously, they convert a part of the produced acids into solvents. Intriguingly, also several other genes, some of which may be involved in the utilization of alternative substrates, revealed the same expression pattern as the cellulosome-like genes and were strongly expressed only during solventogenic steady-state conditions, i.e. the metalloprotease *nrpE* (CAC2517), flavodoxin (CAC3664), a hydrolase (CAC3665), a hypothetical protein (CAC1336), the glycerol-3-phosphate dehydrogenase *glpA* (CAC1322), and the putative transcriptional regulator for xylose breakdown *xylR* (CAC0933).

In contrast to all batch fermentation experiments where solventogenesis was coupled to sporulation, *spo0A* showed no significant changes in transcript levels in our continuous cultures. The concentration of *spo0A* therefore seemed not to be the proclaimed 'master controller' [Scotcher and Bennett, 2008] of the transition from acidogenic to solventogenic growth in continuous cultures. Of the sigma factors, only the *sigF*-operon involved in early forespore-specific sporulation [Paredes et al., 2005], placed in group 2 (fig. 2), was significantly induced during the metabolic switch from acidogenesis to solventogenesis. These genes are known to be positively regulated by Spo0A [Tomas et al., 2003]. The expression patterns of *sigF*, *spo0A*, and enzymes of solventogenesis such as *adc* and the *sol*-operon differed from each other in the continuous culture experiment presented here. Recent transcriptional data from batch fermentations described that the onset of *sigF* expression coincides with the gene expression of *spo0A* and the *sol*-locus [Alsaker and Papoutsakis, 2005]. The transcription of several sporulation genes such as *spoVAE* (CAC2302)–*spoVAC* (CAC2305), *spoIIP* (CAC1276) and the spore coat proteins (CAC1298, CAC1335, CAC3317) was below the detection limit, whereas other genes such as *spo0A, spoIVB* (CAC2072), *spoIIIE* (CAC1812), and *spoVE* (CAC2126) were detectable but not significantly regulated within the experiment (data not shown). These results underscore the experimental separation of solventogenesis and sporulation in the continuous culture.

Besides Spo0A, the transcriptional regulator AbrB has been described to play an important role for the initiation of sporulation and solventogenesis in *C. acetobutylicum* [Scotcher et al., 2005]. In *Bacillus subtilis* the *abrB* gene is expressed during vegetative growth and represses the transcription of sporulation-associated genes such as *spo0A* [Strauch et al., 1989a, b]. Previous data showed that a *C. acetobutylicum* strain containing an antisense RNA construct against *abrB* (CAC0310) generated a twofold higher amount of acetate and butyrate. Moreover, the accumulation of acetone and butanol decreased along with a delay in sporulation [Scotcher et al., 2005]. Our data demonstrated that two *abrB* genes (CAC0310, CAC3647) of *C. acetobutylicum* are significantly expressed throughout acidogenic growth, whereas their gene expression was inhibited during the transition to solventogenesis (fig. 2). This showed that while AbrB may well be a regulator involved in the transition from acidogenic to solventogenic growth, it was absent during solventogenic growth itself.

Our data suggests that the expression of the major stress response genes might be triggered by the decrease of the pH and not by the increased butanol concentration of the medium. On the other hand, it has been reported that challenging *C. acetobutylicum* with different amounts of butanol in the medium, comparable to those found in our chemostat, revealed a concentration-dependent increase of the expression of all major stress response genes [Tomas et al., 2004]. However, former batch fermentations with a starting pH of 6.9 and a maintained pH of 5.0 showed already significant transcription levels of stress response genes during the mid-exponential acidogenic growth and at the beginning of the stationary growth phase, when butanol was still absent [Alsaker and Papoutsakis, 2005]. The results from challenging with butanol were somewhat contradictory to the transcriptional data from continuous cultures presented here. We observed high transcription of the protein stress response genes *ctsRyacH-yacI-clpC*, *hrcA-grpE-*

dnaK-dnaJ, and the *groEL-groES* operon as well as the *hsp18* gene only during the transition from acidogenesis to solventogenesis, when butanol was still absent from the medium. As long as the pH stayed above 5.8, no transcription of the stress response genes occurred. As soon as the pH value dropped below 5.8, the expression of these genes was induced even though butanol was still absent from the medium (fig. 1b, 2). This corresponds to acid stress response in lactic acid bacteria [Frees et al., 2003; Kang et al., 2010]. During solventogenic steady-state conditions at pH 4.5 the transcription of stress response genes was again not significant any longer (fig. 2). Northern blot analysis of *hsp18* during the pH shift in a phosphate-limited chemostat [Sauer and Dürre, 1995] confirmed this expression pattern. These data clearly indicate that butanol at the concentrations observed here seems not to be a trigger or at least not the sole trigger for the stress response.

Recently it was suggested that the regulation of fatty acid biosynthesis is influenced by butanol stress [Tomas et al., 2004]. However, except for the 3-oxoacyl-ACP synthase (CAC0814), all other genes encoded on the large fatty acid synthesis cluster (CAC1988–CAC2019) were already induced during the transition. Because butanol has not yet accumulated at this point of time, these results suggest that in continuous cultures the expression of fatty acid synthesis genes was not directly triggered by butanol stress.

As previously described [Nolling et al., 2001], there are two sets of genes on the chromosome coding for a 3-hydroxybutyryl-CoA dehydrogenase (CAC2708 and CAC2009) and an enoyl-CoA hydratase (CAC2712, CAC2012 and CAC2016). These genes are involved in the conversion of acetoacetyl-CoA to crotonyl-CoA. On the one hand, 3-hydroxybutyryl-CoA dehydrogenase *hbd* (CAC2708) and the enoyl-CoA hydratase *crt* (CAC2712) were significantly induced during acidogenesis and repressed when the pH dropped, on the other hand, *mmgB* (CAC2009) and *fadB* (CAC2012, CAC2016) genes that also code for a 3-hydroxybutyryl-CoA dehydrogenase and enoyl-CoA hydratase became induced when switching from acidogenesis to solventogenesis at pH 5.5. They remained expressed throughout solventogenesis. Therefore, *C. acetobutylicum* seems to use two different sets of genes for the conversion of acetoacetyl-CoA to crotonyl-CoA during acidogenic and solventogenic growth.

The two paralogs of thiolases *thlA* (CAC2873) and *thlB* (CAP0078) as well as the alcohol/aldehyde dehydrogenases *adhE1* (CAP0162) and *adhE2* (CAP0035) were also antagonistically regulated (fig. 7). While *adhE2* was strongly induced during acidogenic growth, the second alcohol dehydrogenase encoded at the *sol*-locus, *adhE1*, was repressed. The contrary expression pattern was observed throughout solventogenic growth. Only a very low amount of *adhE2* transcript was detected at the end of the pH shift and during solventogenic steady-state conditions. These results suggested that *adhE1* is mainly responsible for alcohol production throughout solventogenic growth.

Unlike *adhE1*, which was upregulated during the transition and the solventogenic growth, the second thiolase, *thlB*, was only induced within the switch and exhibited no significant change in expression levels when comparing acidogenic and solventogenic steady-state conditions (fig. 7). These results agreed with transcript levels measured by RT-PCR [Winzer et al., 2000]. We assume that by this expression pattern high levels of acetoacetyl-CoA became available for the activation of free acids from the medium during the switch to solventogenesis (fig. 7).

The gene coding for the pyruvate decarboxylase *pdc* (CAP0025) was only induced during the metabolic shift from acidogenesis to solventogenesis (fig. 2). The enzyme is involved in the formation of ethanol by the cleavage of pyruvate to acetaldehyde and carbon dioxide. Acetaldehyde is subsequently reduced to ethanol via the alcohol dehydrogenase *adhE1* that became induced contemporaneously, whereas the aldehyde-alcohol dehydrogenase *adhE2* became repressed during the transition to solventogenesis (fig. 7). We assume that by this expression pattern, pyruvate coming from glycolysis was channeled to the neutral end-product ethanol. This reduced the feed of C2 units from glycolysis in the acetone- and butanol-forming metabolism when large amounts of extracellular acetate were assimilated during the switch.

The expression of the *sol*-operon during the pH shift was similar to the transcript level observed under solventogenic steady-state conditions as shown in figure 3, group 2 part I. Notice the color change from green (repressed) to yellow (no significant regulation). While in batch cultures of a *buk* (butyrate kinase) mutant the expression pattern of the *sol*-locus (CAP0162–CAP0164) and *adhE2* were described to be similar [Zhao et al., 2005], we found *adhE2* to be expressed antagonistic to the *sol*-operon.

While aconitate hydratase *citB* (CAC0971) and isocitrate dehydrogenase *citC* (CAC0972) were not significantly regulated when comparing acidogenic and solventogenic steady-state conditions, they became strongly re-

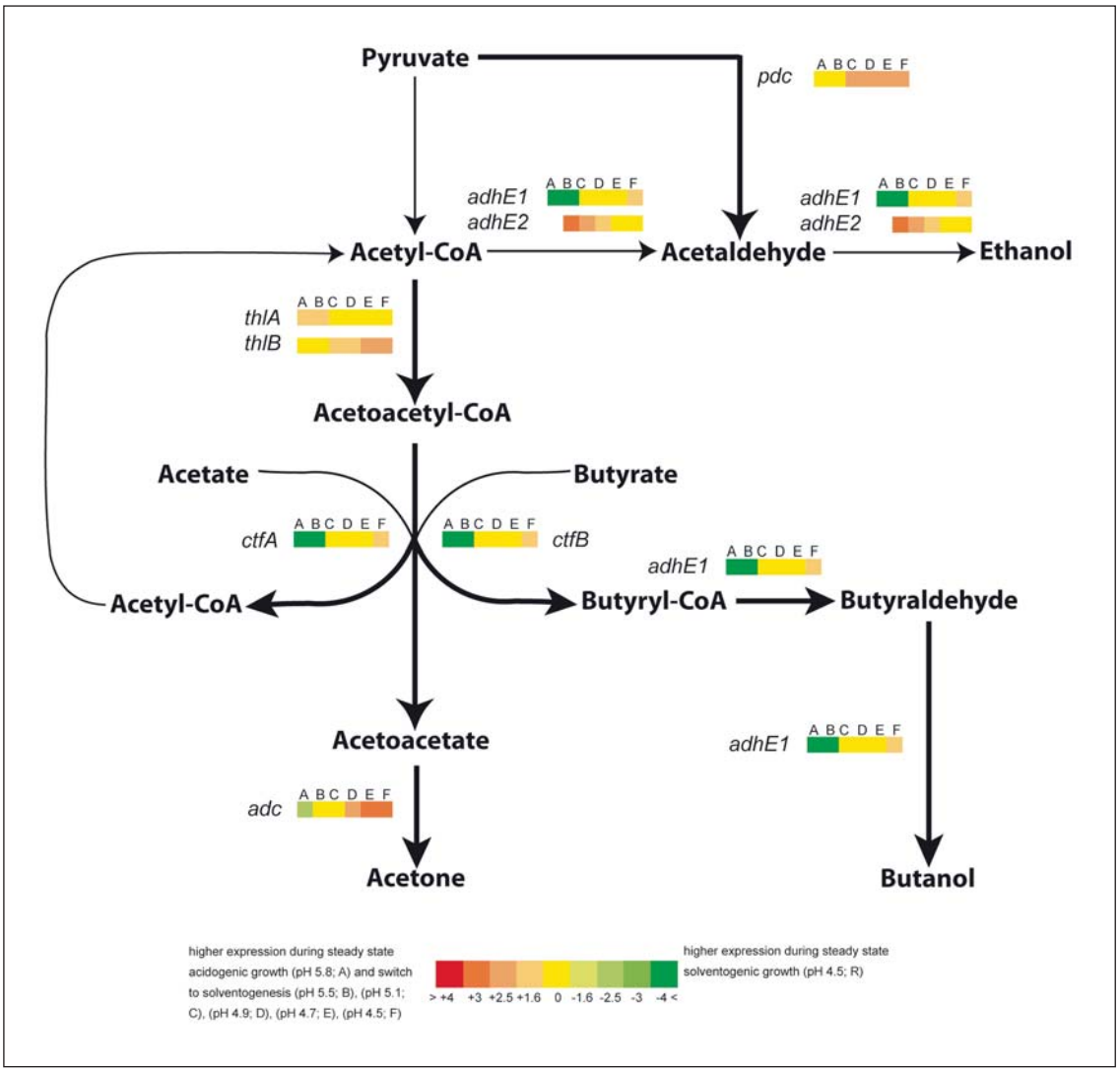


Fig. 7. Solventogenic fermentation pathway. Changes of gene expression during the metabolic switch from acidogenesis to solventogenesis. RNA samples taken at the given pH values (A: pH 5.8 steady state, B–F: 5.5–4.5 dynamic shift) were hybridized against RNA from pH 4.5 steady state (R: common reference). To compare acidogenic and solventogenic steady-state conditions (A/R) the arithmetic mean of the experiment and its dye-flip was calculated. The figure shows ratios as logarithms to the basis of 2. White squares indicate that microarray signals were too close to the background to calculate a ratio.

pressed during the shift to solventogenesis (fig. 3). This could reflect the requirement to use acetyl-CoA for the activation of acids during the initiation of solventogenesis instead of citrate formation.

In conclusion, the continuous culture experiment presented here is in agreement with other continuous culture experiments [Bahl et al., 1982b; Grupe and Gottschalk, 1992]. In addition to results reported from batch culture studies [Alsaker and Papoutsakis, 2005; Jones et al., 2008; Tomas et al., 2003] it allowed insight into the details of the function of isoenzymes for the switch from acidogenesis to solventogenesis. We had shown that the transcriptional regulation of genes concerned with solventogenesis was clearly independent from sporulation and butanol stress. An interesting observation was the expression of the putative cellulosome under solventogenic conditions.

Experimental Procedures

Bacterial Strain and Growth Conditions

For precultures, 5 ml clostridial growth medium (CGM) [Wiesenborn et al., 1988] was inoculated with 500 μl *C. acetobutylicum* ATCC 824 spore suspension and pasteurized for 10 min at 75°C before incubation under anaerobic conditions (N_2) at 37°C. Phosphate-limited continuous culture experiments were performed according to Bahl et al. [1982b] and Fischer et al. [2006] in a one-liter Biostat B*plus* fermenter system (Sartorius BBI Systems, Melsungen, Germany). Phosphate limitation was checked with the Quantofix Phosphate kit (Macherey-Nagel, Düren, Germany). The external pH was regulated automatically by addition of 2 M KOH. Continuous cultures were grown with a medium containing 0.5 mM phosphate at 37°C and 200 rpm. The dilution rate was set to 0.075 h^{-1}. For microarray analysis, cell pellets from 10 ml culture were collected by centrifugation at –20°C and 9,000 rpm (Hettich Universal 320R, Tuttlingen, Germany) for 5 min. Afterwards, pellets were frozen in liquid nitrogen and stored at –80°C. Cell growth was determined by measuring the OD at 600 nm in an Ultrospec 3300 Pro UV/Visible spectrophotometer (GE-Healthcare, Munich, Germany).

Analytical Methods

The concentration of fermentation products (butyrate, acetate, ethanol, acetone and butanol) in the cell-free supernatant was measured by gas chromatography (CP9001; Chrompack, Frankfurt am Main, Germany) using a Chromosorb 101 (80/100 mesh) column according to the method of Thormann et al. [2002]. The residual amount of glucose in culture supernatants was determined by using a Dionex DX-500 high-pressure liquid chromatography system (Dionex GmbH, Idstein, Germany) equipped with a CarboPac PA1 anion-exchange column. Elution was achieved with 250 mM sodium hydroxide at a flow rate of 1 ml/min.

RNA Isolation and Purification

For RNA isolation a washed cell pellet was resuspended in cold TE buffer and immediately frozen in liquid nitrogen. Cells were lysed by grinding the frozen pellet in a chilled Mikro-Dismembrator U ball mill (BraunBiotech, Melsungen, Germany) for 3 min at 1,600 rpm and the resulting powder was resuspended at room temperature in the guanidine-isothiocyanate containing RLT buffer from a RNeasy Midi Kit (Qiagen, Hilden, Germany) ensuring complete inhibition of RNA degradation. After removal of debris by centrifugation and addition of cold ethanol, the supernatant was transferred on an RNeasy column and RNA was purified according to the suppliers' instructions. DNA was removed from the resulting RNA by incubation for 60 min with 100 U of DNAse (RNAse-free). The reaction was stopped by exposure of the samples to 70°C for 10 min. DNAse was removed by phenol and chloroform extraction. RNA was precipitated by addition of sodium acetate and ethanol and washed with 70% ethanol afterwards. The resulting RNA concentration was determined photospectrometrically. The integrity of the RNA was checked by RT-PCR of ATPase genes after ensuring absence of contaminating DNA by PCR and additionally controlled in a BioAnalyzer (Agilent, Böblingen, Germany) run.

DNA Microarray

The *C. acetobutylicum* array was constructed by spotting 5′ amino-C6-modified oligonucleotides with a length of 60–70 bases on CodeLink microarray slides (SurModics, Eden Prairie, Minn., USA) using a MicroGrid II microarray spotter (Zinsser Analytic, Frankfurt, Germany). Oligonucleotides were covalently coupled to the slides surface. The array contained 3,840 oligonucleotides representing 99.8% of all annotated open reading frames (orfs) in *C. acetobutylicum* with one oligo per orf, including all orfs from the pSOL1 megaplasmid.

cDNA Labeling and Hybridization

For preparation of labeled cDNA, 15 μg random hexamer primer were annealed in a volume of 10 μl to 25 μg of RNA by incubation at 70°C for 10 min. Then 1 mM dATP, dTTP, dGTP as well as 0.4 mM dCTP, 50 μM Cy3- or Cy5-labeled dCTP (GE-Healthcare), 10 mM DTT and 200 U SuperScript III reverse transcriptase (Invitrogen, Carlsbad, Calif., USA) were added and the labeling reactions with a total volume of 20 μl were incubated for 2–3 h at 42°C. RNA was removed from the formed heteroduplexes by addition of 2 μl 2.5 M NaOH and incubation for 15 min at 37°C. Hydrolysis was stopped by addition of 10 μl 2 M Hepes pH 7.0. Labeled cDNA was separated from the reaction mixture using GFX columns (GE-Healthcare) according to the suppliers' instructions with the only modification of washing the columns four times before elution of labeled cDNA. Incorporation of Cy-3 or Cy-5 was checked qualitatively by a spectrophotometric wavelength scan and was quantified by using molar extinction coefficients of 150,000 l mol^{-1} cm^{-1} (at 550 nm) for Cy-3 and 250,000 l mol^{-1} cm^{-1} (at 650 nm) for Cy-5. Before hybridization, samples were denatured by incubation at 98°C for 5 min. The hybridization was done in Tom Freeman hybridization buffer [Fitzpatrick et al., 2005] for 15 h at 45°C with cDNA containing approximately 80 pmol of Cy-3 and Cy-5 in an automatic Lucidea slide processor (GE-Healthcare). Slides were washed using a program applying consecutive washes two times with 1× SSC buffer containing 0.2% SDS and then with 0.1× SSC. At the end the hybridization, chambers were flushed with isopropanol and the slides were dried by evaporating the isopropanol with air. Scanning was done using a GenePix 4000B microarray scanner (Molecular Devices, Ismaning, Germany) using GenePix Pro 6.0 software. The specificity of hybridization was checked by control probes with decreasing homology and negative controls.

Microarray Data Analysis

To normalize the data we set the arithmetic mean of the ratios equal to 1 (GenePix Pro 6.0 software). Only features were included in the analysis with fluorescence greater than the local background plus 1 standard deviation. To correct for features with irregular spot morphology, only those were included where the ratio of medians, the ratio of means and a regression ratio differ less than 30%. The transcriptional data shown here are reproduced in two independent biological experiments. Genes were treated as significantly upregulated or repressed if the logarithmic (to the basis of two) expression ratio was ≥1.6 or ≤–1.6. Results were considered as reproduced if the expression ratio in the second experiment was ≥1.3 or ≤–1.3. Exemplary data of one biological experiment are presented. Microarray data were deposited in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under the accession number E-MEXP-2314.

Real-Time PCR

Real-time PCR was performed using SYBR Green I technology on an iCycler iQ real-time PCR detection system (BioRad Laboratories, München, Germany). The reverse transcription of RNA and the following PCR amplification were carried out in one step using iScript One-Step RT-PCR kit with SYBR Green (BioRad Laboratories). One 25-μl PCR reaction consisted of: 12.5 μl 2× SYBR Green RT-PCR reaction mix (including iTaq antibody-mediated hot-start DNA polymerase), 70 μM forward primer, 70 μM reverse primer (online supplementary table 1, www.karger.com/doi/10.1159/000320973), 100 ng template RNA, 0.5 μl iScript reverse transcriptase for one-step RT-PCR. Dynamic well factors were used to compensate for any system non-uniformity in order to optimize fluorescent data quality and analysis. The following PCR program was used: 20 min at 50°C, 10 min at 95°C, 45 cycles 10 s at 95°C and 30 s at 56°C followed by 30 s 72°C, 1 min 95°C and 1 min at 45°C. To verify the specificity of amplified products, a melting-point analysis (45–95°C) was performed after the final PCR step. All samples were amplified in duplicates and the mean value was considered. Negative controls without template were run for each primer. The real-time PCR data were analyzed with iQ5 software (BioRad Laboratories) and relative quantification was done by using the 'delta-delta CT method' [Pfaffl, 2001]. The appropriated housekeeping genes are listed in online supplementary table 1.

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