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BIOENERGY AND BIOFUELS

Comparison of expression of key sporulation, solventogenic and acetogenic genes in *C. beijerinckii* NRRL B-598 and its mutant strain overexpressing *spo0A*

J. Kolek[1] · M. Diallo[2] · M. Vasylkivska[1] · B. Branska[1] · K. Sedlar[3] · A. M. López-Contreras[2] · P. Patakova[1]

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Abstract The production of acetone, butanol and ethanol by fermentation of renewable biomass has potential to become a valuable industrial process. Mechanisms of solvent production and sporulation involve some common regulators in some ABE-producing clostridia, although details of the links between the pathways are not clear. In this study, we compare a wild-type (WT) *Clostridium beijerinckii* NRRL B-598 with its mutant strain OESpo0A, in which the gene encoding Spo0A, an important regulator of both sporulation and solventogenesis, is overexpressed in terms of solvent and acid production. We also compare morphologies during growth on two different media: TYA broth, where the WT culture sporulates, and RCM, where the WT culture does not. In addition, RT-qPCR-based analysis of expression profiles of *spo0A*, *spoIIE*, *sigG*, *spoVD*, *ald* and *buk1* genes involved in sporulation or solvent production in these strains, were compared. The OESpo0A mutant did not produce spores and butanol titre was lower compared to the WT, but increased amounts of butyric acid and ethanol were produced. The gene *spo0A* had high levels of expression in the WT under non-sporulating culture conditions while other selected genes for sporulation factors were downregulated significantly. Similar observations were obtained for OESpo0A where *spo0A* overexpression and downregulation of other sporulation genes were demonstrated. Higher expression of *spo0A* led to higher expression of *buk1* and *ald*, which could confirm the role of *spo0A* in activation of the solventogenic pathway, although solvent production was not affected significantly in the WT and was weakened in the OESpo0A mutant.

Keywords *Clostridium* · *spo0A* · Butanol · qPCR · Overexpression · ABE · Sporulation

Introduction

The acetone-butanol-ethanol fermentation (ABE) process is a promising option for production of biochemicals and biofuels from renewable resources in a future biorefinery. Although butanol is the main product of the ABE process, other metabolites, acetone, organic acids or hydrogen also represent valuable chemicals that may increase the attractiveness of this technology (Sauer 2016). Unfortunately, there are still obstacles preventing implementation of industrial large-scale production of butanol or other products by ABE fermentation, primarily a poor understanding of process regulation, making substantial improvements in this technology difficult to achieve. Solventogenic strains of *Clostridium* sp., performing ABE fermentation, are Gram-positive strictly anaerobic microorganisms with a complex cell cycle including endospore formation. The regulation of sporulation includes the activation or repression of genes that are expressed sequentially during the course of the sporulation cascade. The sporulation mechanism in clostridia differs significantly from that described for the *Bacillus* model and for some steps, details of

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✉ P. Patakova
petra.patakova@vscht.cz

[1] Department of Biotechnology, University of Chemistry and Technology Prague, Technicka 5, 166 28 Prague, Czech Republic

[2] Wageningen Food and Biobased Research, Wageningen University and Research, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands

[3] Department of Biomedical Engineering, Brno University of Technology, Technicka 12, 61600 Brno, Czech Republic

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the cascade remain unclear (Al-Hinai et al. 2015). Furthermore, some sporulation regulatory factors, especially the main regulator Spo0A, may have a major impact on other cellular functions, including solventogenesis. Despite progress in genetic engineering of solventogenic clostridia, as well as the successful implementation of modern "omics" methods in clostridial research over the last decade, the interconnection between sporulation and solventogenesis is still unexplained.

The Spo0A protein is a master transcriptional regulator in all known endospore formers (Brown et al. 1994). In *Bacillus* sp., Spo0A is activated by phosphorylation, mediated by KinA and KinB orphan kinases through activation of Spo0F and Spo0B proteins, and activation of the sporulation cascade is initiated by starvation or the recognition of an unfavourable environment (Piggot and Hilbert 2004). In clostridia, the mechanism of Spo0A phosphorylation is in dispute (Al-Hinai et al. 2015), but most probably some genomic orphan histidine kinases are likely to be involved in activation of the Spo0A regulator (Steiner et al. 2011; Mearls and Lynd 2014). A lack of nutrients generally prevents sporulation in clostridia, a property that is also in variance with *bacilli* (Jones and Woods 1986).

The activated Spo0A protein is able to regulate expression of different genes. Spo0A-P together with σ^H activates the *spoIIA* operon, including the first pre-spore specific sigma factor σ^F, which initially is blocked by anti-sigma factor SpoIIAB. For σ^F activation, membrane-bound SpoIIE factor is necessary. Activation of σ^F leads to the activation and expression of about 50 specific genes and operons in the pre-spore, including the SpoIIR regulator that activates expression of the *sigE* operon (Higgins and Dworkin 2012; King et al. 1999; Al-Hinai et al. 2015). Active σ^E (encoded by the *sigE* gene) is located in the mother cell and controls expression in this compartment, especially during sporulation stage II. σ^G (encoded by the *sigG* gene) is a second pre-spore specific sigma factor that is expressed and activated directly after σ^F. Factor σ^G is the main regulator of sporulation stage III and controls expression of many pre-spore specific genes and operons; some of them are involved in activation of the last sigma factor σ^K. Expression of this last, mother cell located sigma factor starts during sporulation stage III and regulates all remaining reactions necessary for maturation and release of an endospore from the mother cell, including genes guiding spore cortex and coat synthesis, e.g. SpoVD, CotJ, CotJC and many others (Al-Hinai et al. 2015).

C. beijerinckii NRRL B-598, previously *C. pasteurianum* (Sedlar et al. 2017), is a solventogenic strain suitable for production of butanol, acetone or hydrogen (Lipovsky et al. 2016) and which excels in oxygen resistance (Kolek et al. 2016b) and overall robustness. The complete genomic sequence (Sedlar et al. 2015) and transformation methods (Kolek et al. 2016b) are also available for this strain. In this work, expression of the *spo0A* global regulator and its impact on the expression of genes selected from different stages of the sporulation cascade (*spoIIE*—stage II; *sigG*—stage III; *spoVD*—stage V), as well as genes involved directly in solventogenesis (*ald*, coding an aldehyde dehydrogenase) and acetogenesis (*buk*, coding a butyrate kinase), were investigated in cultures showing different patterns of sporulation. The genes of interest were selected to cover different periods of the cell cycle, i.e. acidogenesis, solventogenesis and different phases of sporulation, because Spo0A is a global transcriptional regulator that may affect expression of individual genes, both directly and indirectly. Based on previous experience (Kolek et al. 2016a), two different cultivation strategies were used for solvent production: with and without spore formation. Furthermore, the same analysis was performed for a novel strain, *C. beijerinckii* NRRL B-598 OESpo0A, bearing simple overexpression of the *spo0A* regulator. In this work, we have extended knowledge on the regulation of sporulation and solventogenesis beyond that of the well-described strains *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052.

Material and methods

Bacterial strains, growth and maintenance conditions

Solventogenic bacterium *C. beijerinckii* NRRL B-598 (NRRL/ARS Culture Collection, Peoria, Illinois, USA) and mutant strain *C. beijerinckii* B-598 OESpo0A (this article) were used in this study. The WT was maintained as a spore suspension in distilled water, OESpo0A strain was cryopreserved in 30% glycerol and − 80 °C. TYA medium (containing in g/l: 50 glucose; 2 yeast extract (Merck, Darmstadt, Germany); 6 tryptone (Sigma-Aldrich, St.Louis, Missouri, USA); 0.5 KH_2PO_4; 3 ammonium acetate; 0.3 $MgSO_4.7H_2O$; 0.01 $FeSO_4$) and modified RCM broth (containing in g/l: 50 glucose; 10 meat extract (Merck, Darmstadt, Germany); 10 peptone (Sigma-Aldrich, St.Louis, Missouri, USA); 3 yeast extract (Merck, Darmstadt, Germany); 1 starch; 5 sodium chloride; 3 sodium acetate; 0.5 L-cysteine hydrochloride) were used for growth and fermentation of microorganisms and were supplemented with erythromycin (15 μg/ml) in the case of strain OESpo0A.

Escherichia coli strains DH5α and JM110 (both purchased from the Leibnitz Institute DSMZ- German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cryopreserved in 20% glycerol solution (maintained in − 80 °C) and grown on LB medium (containing in g/l: 10 tryptone; 5 yeast extract; 5 NaCl) in 37 °C. LB broth or plates (1.5% agar) were supplemented with erythromycin (500 μg/ml) if necessary.

Plasmids, oligonucleotides and DNA manipulation

High pure PCR template preparation kit (Roche Diagnostics, Basel, Switzerland) was used for preparation of the template for PCR reactions and high pure plasmid isolation kit (Roche Diagnostics, Basel, Switzerland) was used for all plasmid isolations from *E. coli* strains; plasmid extraction from *C. beijerinckii* was performed as we described previously (Kolek et al. 2016b). For PCR reactions, gbELITE MasterMix was used (Generi-Biotech, Hradec Kralove, Czech Republic). Restriction enzymes, T4 ligase and kit for DNA isolation from an agarose gel were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). DNA was introduced into *E. coli* cells by electroporation with the GenePulser Xcell™ electroporator (BioRad, Hercules, California, USA) using conditions for *E. coli* pre-set by the manufacturer and using 0.1-cm gap electroporation cuvettes. Transformation of *C. beijerinckii* NRRL B-598 was carried out by sono/electroporation as described previously (Kolek et al. 2016b).

Preparation of *C. beijerinckii* B-598 OESpo0A and control strain

The *spo0A* gene was amplified by PCR from the chromosome of *C. beijerinckii* NRRL B-598 with primers OESpo0A_*Sac*I_addF (5′-TACAGTGAGCTCAAAAAATA TGGAAGATTC–3′) and OESpo0A_*Xma*I_addR (5′-TAGT ATCCCGGGTACATAAAAAAAAGACC-3). The product obtained, including an extra region of 12 nt downstream and 463 nt upstream, was isolated from an agarose gel, digested by *Sac*I and *Xma*I and cloned into the pMTL83253 plasmid (Heap et al. 2009) digested by the same enzymes. The resulting plasmid pMTL83253::OESpo0A was transformed into *E. coli* DH5α and control restriction digestion was performed. Plasmid pMTL83253::OESpo0A was subsequently extracted and transformed into *E. coli* JM110 (dam−/dcm−) and extracted for *C. beijerinckii* NRRL B-598 transformation. Plasmid extraction and control restriction digestion by *Sac*I/*Xma*I and *Sma*I/*Xmn*I were performed for several erythromycin resistant colonies. The control strain was prepared by transformation of pMTL83253 into *C. beijerinckii* NRRL B-598.

Cultivation in bioreactor

Parallel Multiforce 2 bioreactors (Infors HT, Bottmingen, Switzerland) were used. Cultivations were performed at 37 °C with 360 ml TYA or RCM broth without pH control during fermentation and agitation at 250 rpm. Oxygen was removed from bioreactors by bubbling with N_2 for 30 min and pH was adjusted to 6.3–6.4 using 10% NaOH if necessary prior to cultivation. All bioreactors were inoculated with 40 ml of inoculum that was cultured previously in an anaerobic chamber overnight (Concept 400; Baker Ruskinn Technology, Bridgend, UK) under a stable anaerobic atmosphere (90% N_2, 10% H_2). Samples were taken at specific times (in hours: 6; 11; 21; 26 and 48) and processed for gravimetric analysis, HPLC analysis, microscopy and RNA isolation (except for time 48).

Microscopy

Phase contrast microscopy (Olympus BX51; Olympus, Tokyo, Japan) was used to determine the morphological status of cells and the presence of spores or pre-spore stages. Native bacteria were observed by phase contrast microscopy at ×400 and ×1000 magnifications.

Growth measurement and HPLC analysis

Cell concentration was determined gravimetrically as biomass dry weight. Dry weight was measured from 2 ml samples that were centrifuged (×13.000*g*, 4 °C, 2 min), washed with deionised water, and dried at 105 °C until constant weight was achieved. Results represent average values of three measurements.

The concentrations of glucose, butyric acid, acetic acid, lactic acid, formic acid, ethanol, acetone and butanol were measured in the supernatant of culture broth by HPLC. Samples were diluted 1:1 with internal standard solution (100 mM valeric acid in 1M H_2SO_4) and microfiltered. Analyses were carried out using a Waters HPLC system (Waters, Milford, Massachusetts, USA) equipped with an autosampler using a Shodex KC-211 column (Shodex, Tokyo, Japan) and refractive index (Waters model 2414) as well as UV absorbance (Waters model 2487) detectors. Conditions of analysis were as follows: column temperature 80 °C, mobile phase 3 mM H_2SO_4, flow rate 1 ml/min. The concentrations of glucose, ethanol, acetone and butanol were determined using a refractive index detector, and acid concentrations were determined at 210 nm by UV detector.

RNA isolation

Samples of culture broth for RNA isolation were centrifuged (×13.000*g*, 4 °C, 2 min), washed with chilled RNase free water and suspended in RNase free water to obtain a suspension having an optical density (measured at 600 nm) of approximately one immediately after collection. A 3-ml diluted sample was centrifuged and the cell pellet was stored in − 80 °C for subsequent isolation.

Frozen samples were thawed on ice and RNA was isolated using high pure RNA isolation kit (Roche Diagnostics, Basel, Switzerland). Quality and concentration of RNA samples were checked using a nanodrop machine (Thermo Fisher

Scientific, Waltham, Massachusetts, USA); RNA quality and integrity were also determined using the Agilent RNA 6000 Nano kit (Agilent, Santa Clara, California, USA) with the Agilent 2100 bioanalyzer (Agilent, Santa Clara, California, USA).

Reverse transcription and RT-qPCR analysis

For reverse transcription of RNA samples, the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Basel, Switzerland) was used. Concentration and quality of cDNA samples were checked using a nanodrop machine (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

RT-qPCR analyses were performed with BioRad CFX 96 Touch™ (BioRad, Hercules, California, USA) using a Universal Probe Library (Roche, Switzerland) system of probes and FastStart Universal Probe Master (ROX) mastermix (Roche Diagnostics, Basel, Switzerland); Universal ProbeLibrary Assay Design Center software (https://lifescience.roche.com) was used for oligonucleotide and probe design (all oligonucleotides and probes used in this study are shown in Table 1). Reactions were performed in an overall volume 10 μl with concentrations of components and reaction conditions as described in the mastermix protocol.

Relative expression of genes *spo0A*, *spoIIE*, *sigG*, *spoVD*, *ald* and *buk1* were monitored; gene *rpsJ* was chosen as the reference gene from a selection of candidate genes (including *gyrA*, *16S rrn*, *adk* and *alaS*) based on analysis by RefFinder algorithms. Reaction efficiency was determined for each assay using a ×5 serial dilution of cDNA samples, see Table 1. A sample taken at 6th hour of cultivation in TYA medium was chosen as a calibrator and a reference value for calculation of relative fold expression of each gene in other samples. All RT-qPCR analyses were performed in triplicate using no-template as well as no-RT controls.

Bioinformatics analysis

PromoterHunter at psiSITE (Klucar et al. 2010) was used to predict promoter regions of the genes studied. Further identification and analyses of DNA regulatory motifs were provided by DMINDA (Yang et al. 2017).

Results

Construction of the OESpo0A mutant

Plasmid pMTL83253::OESpo0A harbouring the *spoA* gene under the control of the constitutive Pfdx promoter was constructed. For plasmid construction details, see Fig. S1. Plasmid pMTL83253 (for control strain) or pMTL83253::OESpo0A (for OESpo0A mutant strain), extracted from a Dam−/Dcm− *E. coli* strain, was transformed into WT *C. beijerinckii* NRRL B-598 using a previously described method developed for this microorganism. More than a hundred erythromycin resistant colonies were obtained after transformation and five random colonies of each transformant were screened for plasmid sequence by extraction and specific restriction cleavage. All picked colonies contained the correct plasmid and one of each (mutant and control) was used in subsequent experiments.

Several attempts to construct *C. beijerinckii* NRRL B-598 with *spo0A* gene disruption were also performed previously. Unfortunately, no colonies were obtained during these experiments.

Fermentation performance and phenotype of *C. beijerinckii* NRRL B-598 WT and OESpo0A mutant

Profiles of fermentations carried out with the WT, see Fig. 1, in TYA and RCM media were very similar to each other in terms of solvent and acid production; 9.8 and 9.1 g/l in TYA and RCM media respectively after 24 h cultivation. Butanol

Table 1 Oligonucleotides used in RT-qPCR analyses, reaction efficiencies and homology of the analysed genes with those in *C. beijerinckii* NCIMB 8052 and *C. acetobutylicum* ATCC 824

Target gene [GenBank locus tag in reference sequence NZ_CP011966.2]	Left primer	Right primer	UPL #	PCR eff.	Homology with *C. beijerinckii* NCIMB 8052 (%)	Homology with *C. acetobutylicum* ATCC 824 (%)
spo0A [X276_RS08400]	ccaaataacctgttaatcgcttc	tgaaagagcaataagacatgcaa	144	1.980	100	75
spoIIE [X276_RS00600]	agcactgctcaatagagaaactatgt	aaagcggtagggttaagattagatt	17	2.011	99	59
sigG [X276_RS05925]	cctttataaaagtttctcttgcgttc	tggcgttaatacttcaaaactcc	78	1.952	100	84
spoVD [X276_RS08115]	gggaatacttctgctccatgc	gtggaggttcattaaaagttgga	67	1.954	98	63
ald [X276_RS20360]	accagccttttctatatcagcagt	tctggtaagaaagctataggtgctg	138	1.961	98	Not relevant[a]
buk1 [X276_RS01200]	agcaccttgagctaaagcaagt	tccagaccttaaagcaaaagttg	45	1.970	99	64
rpsJ [X276_RS00930]	aatagctcctgcttgacctgtaa	tggaactaaggatagatttgacgtt	77	2.013	100	85

[a] In *C. acetobutylicum*, there is a different gene, *adhE*, in the sol operon

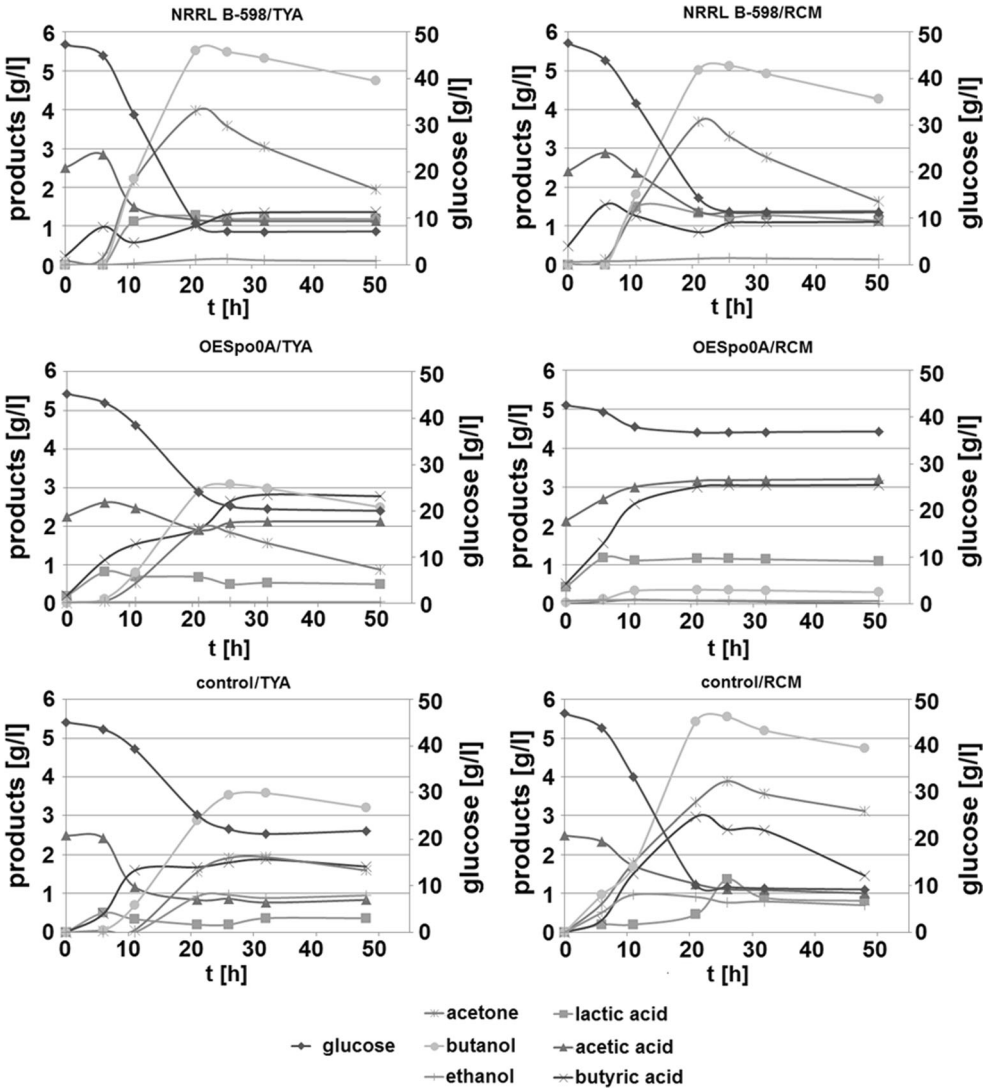

Fig. 1 Fermentation characteristics of *C. beijerinckii* NRRL B-598, control strain and OESpo0A mutant strain using TYA and RCM media; standard deviations of substrate and products HPLC analyses did not exceed 5%

and acetone were detectable in the medium from ca. the 6th hour of cultivation in both media and production of organic acids and glucose consumption were similar, see Fig. 1 and Table 2. Biomass production was also nearly equivalent but from pH monitoring it was apparent that the second pH decrease was slower in RCM, see Fig. 2, which is probably connected with slower acid reutilization.

Motile, rod-shaped cells were observed in both media at the 6th hour, see Fig. 3. In TYA medium, the start of sporulation was clearly observed as a thickening of cells, which is a typical sign of granulose accumulation (Robson et al. 1974). Cell thickening was visible from the 11th hour. From the 21st hour of cultivation, mature spores and pre-spores were observed. On the other hand, no cell thickening or other stages of sporulation were observed in the RCM culture.

C. beijerinckii strain B-598 OESpo0A and the control strain harbouring the parental plasmid (pMTL83253) were cultured in parallel with the WT, see Fig. 1. Butanol and acetone productions in the control strain were overall slightly higher in RCM compared to WT (cf right upper

Table 2 Comparison of fermentation parameters

Strain	Glucose consumption rate g/l h		Butanol productivity g/l h	
	TYA	RCM	TYA	RCM
WT	1.54	1.50	0.24	0.22
OESpo0A	0.92	0.42	0.13	0.01
Control strain harbouring pMTL83253 plasmid	0.95	1.50	0.16	0.24

Both glucose consumption rate and butanol productivity were calculated for the period of first 24 h of respective cultivations

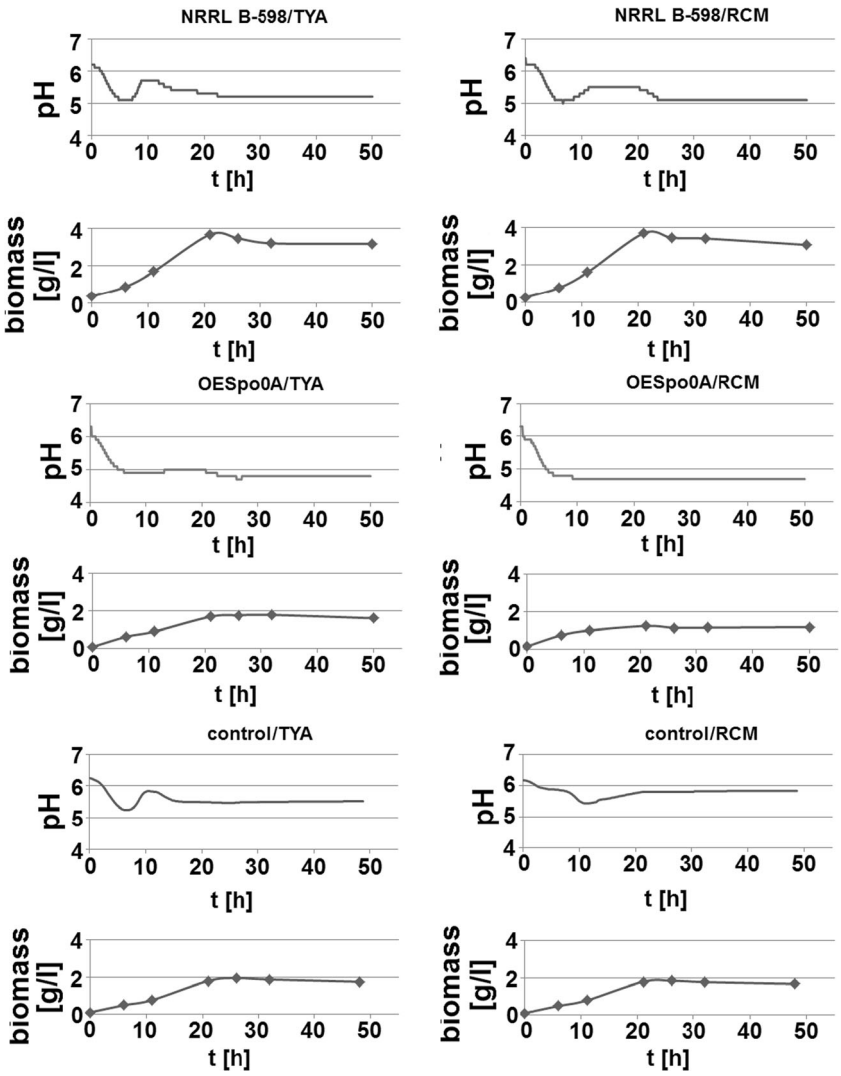

Fig. 2 Course of pH and growth curve represented by dry biomass concentration during cultivation of *C. beijerinckii* NRRL B-598, control strain and OESpo0A mutant strain using TYA and RCM media

graph with right lower graph in Fig. 1), while production of butanol and acetone were reduced significantly in TYA medium (cf left upper graph with left lower graph in Fig. 1). Butyric acid and ethanol productions in the control strain (were greater than WT in both media (cf upper and lower graphs in Fig. 1). The mutant strain, OESpo0A, produced lower titres of solvents and exhibited higher production of butyric acid and a lower consumption of acetic acid compared to the control strain (cf middle and lower graphs in Fig. 1). The maximal concentrations of biomass produced in both media were similar for the control strain and OESpo0A and was ca. two times lower than in the WT, see Fig. 2. The control strain, produced spores only in TYA medium and not in RCM, which is consistent with WT strain results. No mature spores, pre-spores or thickened cells were identified in any of the OESpo0A cultures, grown on either TYA or RCM media, see Fig. 3.

Bioinformatics analysis

Genes participating in different stages of the sporulation cascade, *spo0A*, *spoIIE*, *sigG* and *spoVD* (see Introduction), were selected for monitoring sporulation in this work. Gene *ald* was selected as a marker gene involved in butanol (solvent) production and *buk1* for monitoring of butyrate (acid) production. As the strain studied, *C. beijerinckii* NRRL B-598, is not normally used to investigate butanol production, similarities in all gene sequences with those occurring in the more well studied strains *C. beijerinckii* NCIMB 8052 and *C. acetobutylicum* ATCC 824, are given in Table 1.

Polypeptide sequences of the Spo0A master regulator were almost the same in strains *C. beijerinckii* NRRL B-598 and *C. beijerinckii* NCIMB 8052, differing in only a single amino acid, and the binding motif, helix-turn-helix, sequence was the same in both strains. This motif is also shared by

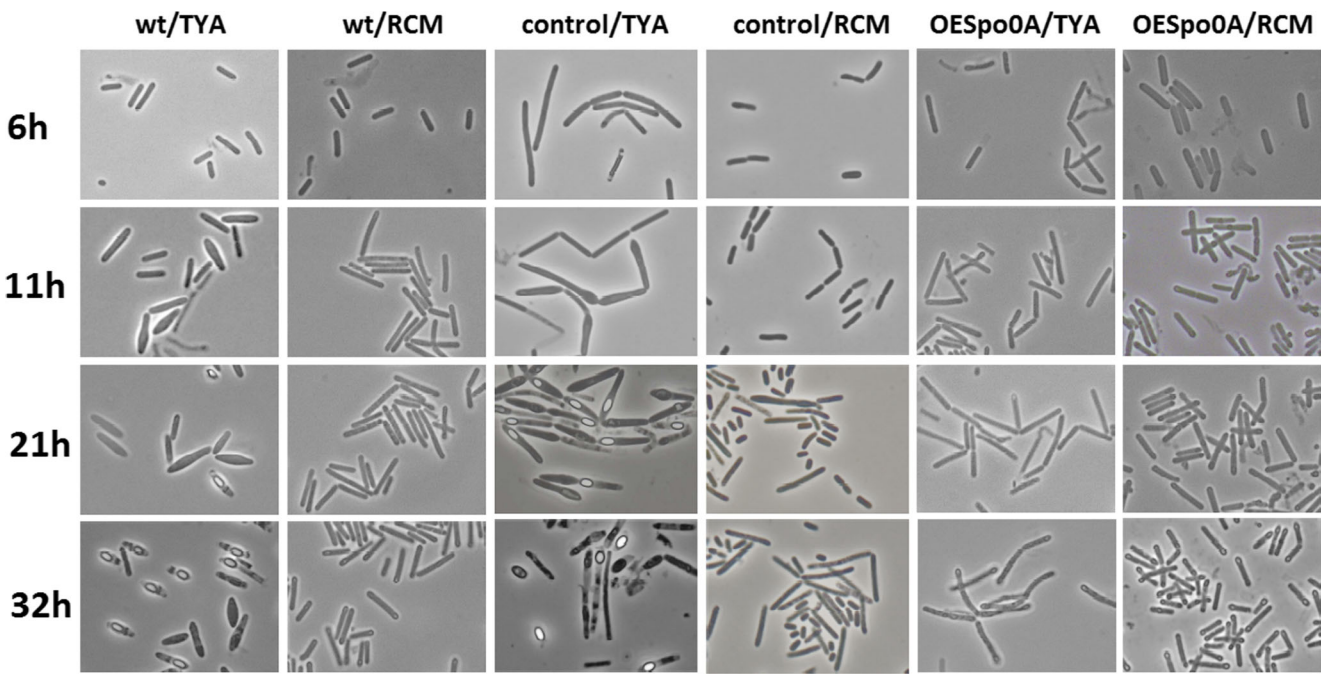

Fig. 3 Morphology of *C. beijerinckii* NRRL B-598, control strain and OESpo0A mutant cells in the course of fermentation in TYA and RCM media

C. acetobutylicum ATCC 824, which showed only 75% similarity with the Spo0A polypeptide sequence from *C. beijerinckii* strains and which is coded for by the antisense strand.

The genes *spoIIE*, *sigG* and *ald* are believed to be directly controlled by Spo0A. The *SpoIIE* sequence of *C. beijerinckii* NRRL B-598 and NCIMB 8052 strains share 99% homology. Moreover, the *SpoIIE* promoter sequence was the same in both strains and contained almost ideal consensual bacterial − 35 (TTGACA) and − 10 (TATAAT) boxes. The sequence between boxes was unusually long, consisting of 21 bp, and the whole promoter ended 25 bp upstream of the transcriptional start site (TSS). In contrast, the sequence preceding the promoter did not contain any putative A0 boxes, for *C. beijerinckii* or *C. acetobutylicum*, defined by the consensus sequence TGNCGAA (Ravagnani et al. 2000), nor the A0 boxes for *B. subtilis* (York et al. 1992). On the contrary, the sequence TTTGGAA, which could also be the A0 box, was found between the promoter and TSS.

The *SigG* gene of *C. beijerinckii* strains shared relatively high (77%) homology with *C. acetobutylicum* and was carried by the sense strand. In both species, the gene has its own promotor but is also the most distant gene of the *spoIIG* operon that is regulated by its promoter. The putative promoter sequence of both *C. beijerinckii* NRRL B-598 and NCIMB 8052 strains was the same and shared only 55% homology with *C. acetobutylicum*. Although two putative mutated binding sites (AACGACA, TTTAACA) were located upstream of the promoter in *C. acetobutylicum*, no statistically significant hit was found for *C. beijerinckii* species.

The remaining gene under direct regulation, *ald*, is a part of the *sol* operon. In *C. acetobutylicum* ATCC 824, this operon was located on a plasmid, and the *ald* gene was replaced by the *adhE* gene. In both *C. beijerinckii* NRRL B-598 and NCIMB 8052 strains, the *sol* operon was carried by the chromosomal sense strand. While the *ald* gene of both *C. beijerinckii* strains shared 97% homology, the promoter region was more variable with overall 95% homology and did not contain any A0 boxes corresponding to the conserved TGNCGAA motif. However, in both *C. beijerinckii* strains, putative A0 boxes differing in the second, fourth and fifth positions were found upstream of the promoter sequence. The sequences of *spoVD* and *buk1* were 98 and 99% homologous, respectively with *C. beijerinckii* NRRL B-598 and NCIMB 8052 strains, as were their putative promoter sequences, although putative 0A boxes were not found. Homologies with *C. acetobutylicum* genes was lower, reaching 62 and 71%, respectively.

Expression of genes involved in sporulation and acids/solvent production in WT

In contrast with production parameters, expression levels of monitored genes differed in TYA and RCM, see Fig. 4. Expression of the Spo0A regulator gene was evident from the first sample, at the 6th hour of cultivation; increase expression of *spoIIE* and *sigG* occurred in mid cultivation and expression of *spoVD* in the last sample—26th hour. The gene for ald, which is involved in the last steps of butanol production in *C. beijerinckii* species, was expressed at the 6th hour and the

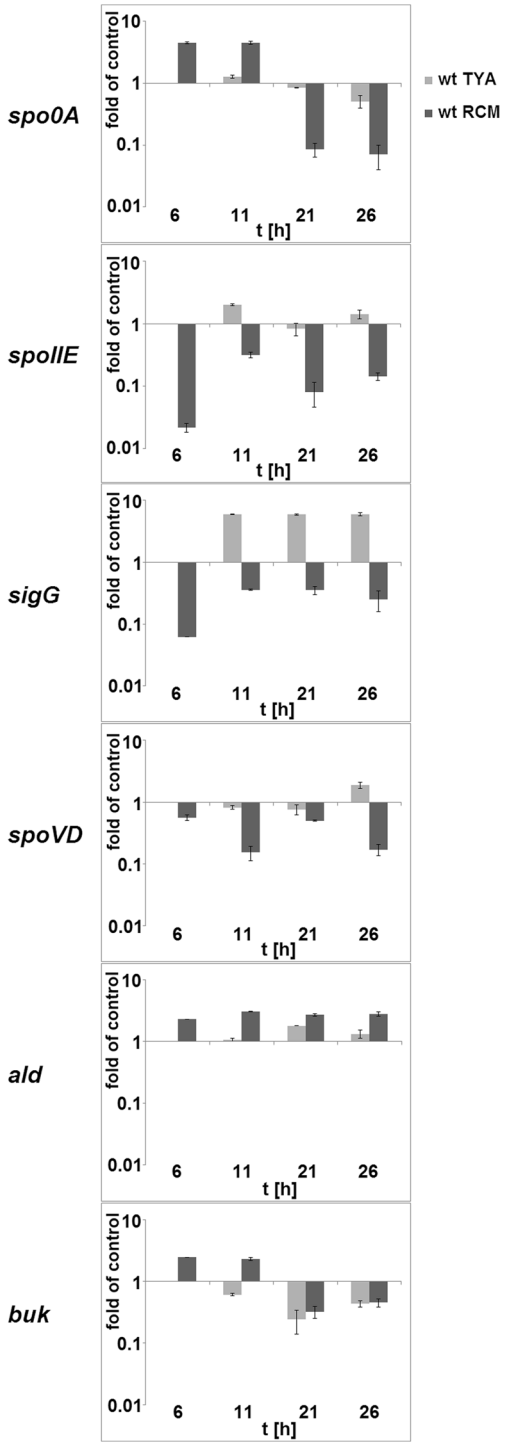

Fig. 4 Relative expressions of monitored genes in WT as obtained by RT-qPCR analysis in the course of cultivation in TYA and RCM media. All relative expression data are related to expression found during cultivation of WT in the 6th hour of cultivation in TYA

next slight increase in expression level was evident at the 21st hour when the concentration of butanol was maximal. Butyrate kinase (*buk1*) was more expressed in the first samples and showed decreased expression during the course of cultivation, which was consistent with maximal butyrate production. Very surprisingly, *spo0A* was significantly more expressed in RCM compared to TYA at the beginning of the cultivation, despite the fact that no spores, pre-spores or thickened cells were identified in RCM culture (see above). All other monitored sporulation factors were strongly downregulated in comparison with TYA culture as well as *spo0A* at 21 and 26 h and slight overexpression of *ald* and *buk1* was recorded in RCM.

Gene expression in OESpo0A

The OESpo0A mutant, overexpressing the *spo0A* gene, showed a deficient sporulation pattern. To determine the effect of overexpression of *spo0A* on the regulation of sporulation-related genes, qPCR analysis of expression of the sporulation genes monitored previously in the WT was performed. As expected, the *spo0A* regulator gene was significantly overexpressed in all samples compared to WT and control strains, see Fig. 5. In TYA medium, expression of monitored genes were quite similar in WT and control strains. Sporulation factors *spoIIE*, *sigG* and *spoVD* were significantly repressed in OESpo0A, which was surprising but fully consistent with our observations obtained in RCM culture of the WT strain.

The *ald* and *buk1* genes were also analysed by qPCR. They were both overexpressed compared to WT in TYA but had a similar trend as in RCM culture. Similarly to WT cultivation, OESpo0A in RCM exhibited relatively higher expression of *spo0A* compared to TYA.

Discussion

As described previously (Kolek et al. 2016a), *C. beijerinckii* NRRL B-598 is able to form solvents in both TYA and RCM broths, but spores are formed only in TYA medium. It is not clear what factors block sporulation in RCM broth but very high concentrations of nutrients, especially sources of nitrogen, or different levels of some microelements such as P, Mg and Fe (Lee et al. 1978), could play some role in this phenomenon. Buffering capacity and the buffering system used was also shown previously to be an important property of the medium, influencing sporulation significantly (Long et al. 1983).

It was also surprising that the control strain of *C. beijerinckii* NRRL B-598 bearing parental pMTL83253 produced significantly more ethanol compared to WT. This was unexpected for this strain although the usual levels of ethanol production by the WT are very low (Lipovsky et al. 2016) and different behaviours of the WT and plasmid-bearing strains have been described previously (Harris et al. 2002; Alsaker et al. 2004).

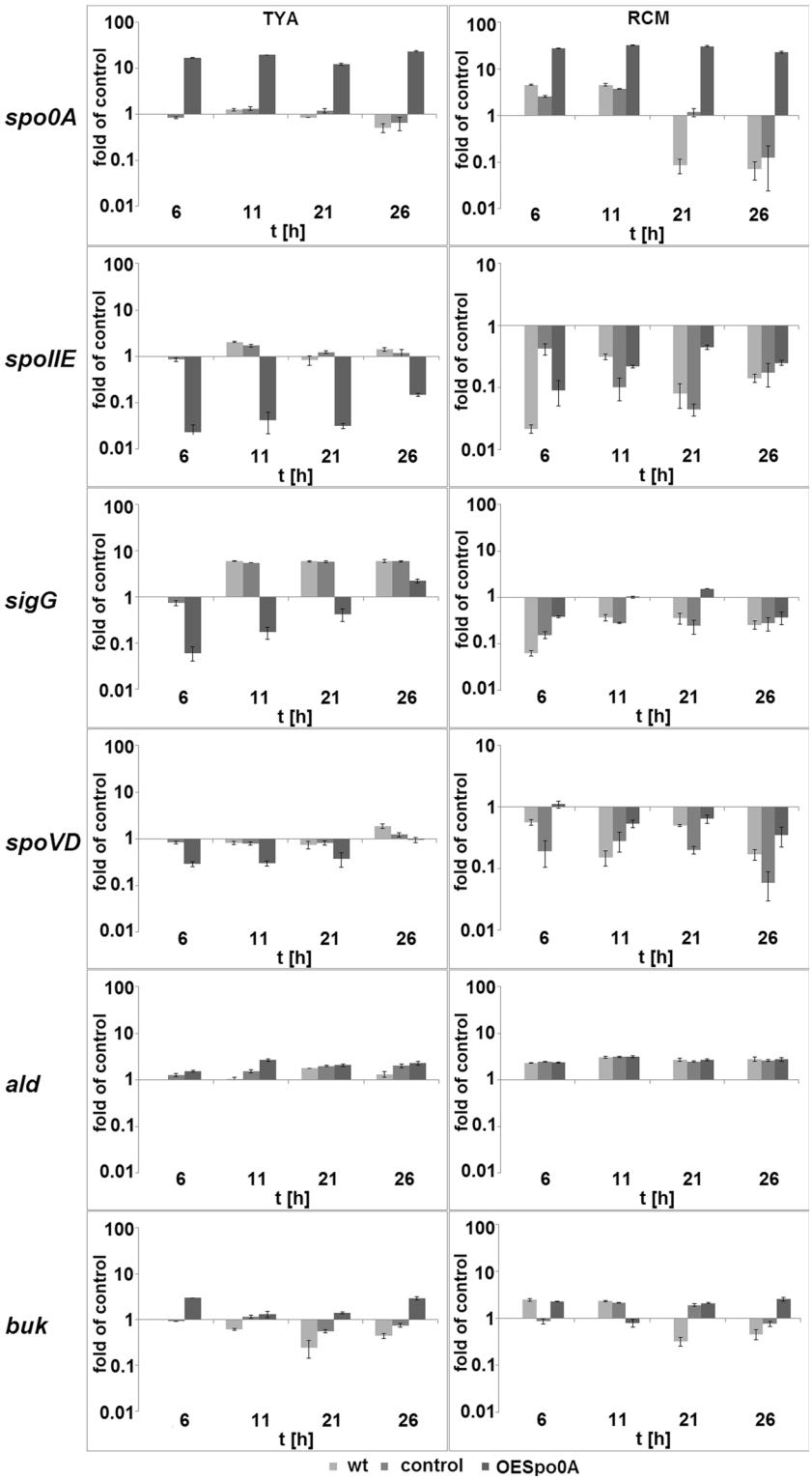

Fig. 5 Relative expressions of monitored genes in WT, control bearing parental pMTL83253 and OESpo0A mutant strain as obtained by RT-qPCR analysis in the course of cultivation in TYA (first column) and RCM media (second column). All relative expression data are related to expression found during cultivation of WT in the 6th hour of cultivation in TYA

Expression of monitored genes at chosen sampling times of WT, OESpo0A and control strain cultivation were measured in this work. Strong overexpression of *spo0A* was confirmed in OESpo0A, which contained a second copy of the *spo0A* gene under the regulation of a strong and constitutive ferredoxin promoter. Expression profiles of *spoIIE*, *sig G* and *spoVD* from the WT strain cultured in TYA medium (the sporulating phenotype) are in close agreement with those observed for both *C. acetobutylicum* ATCC 824 (Alsaker and Papoutsakis 2005; Jones et al. 2008) and *C. beijerinckii*

NCIMB 8052 (Wang et al. 2012). However, in RCM, the WT strain exhibited a different non-sporulating phenotype with different expression profiles of the genes. Nevertheless, a non-sporulating but butanol-producing phenotype was also expressed by *C. acetobutylicum* ATCC 824 under phosphate-limited continuous cultivation (Bahl et al. 1982; Grimmler et al. 2011).

Spo0A represents a conserved regulatory protein that is a master regulator of endospore formation and which is activated by phosphorylation in the cells (Al-Hinai et al. 2015). The Spo0A polypeptide sequence was 75% conserved between *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NRRL B-598 and it is necessary to consider that even a change in a single residue may affect the Spo0A binding affinity (Hatt and Youngman 2000). Activated Spo0A is able to bind many regulatory regions in the genome and can positively as well as negatively affect transcription of a number of genes. Regions binding activated Spo0A contain so-called 0A boxes, the specific motifs of which are recognised by phosphorylated Spo0A (Brown et al. 1994; Ravagnani et al. 2000). Searching for 0A boxes, potential binding sites for Spo0A, in *C. acetobutylicum* ATCC 824 (Sullivan and Bennett 2006) revealed many mutated, reversed or multiple 0A boxes in upstream regions of other genes or operons that are not closely related to sporulation or solventogenesis; this confirms the position of Spo0A as a global transcriptional regulator. Sporulation is blocked in the case of disruption of *spo0A* (Al-Hinai et al. 2015; Huang et al. 2004), but its influence on other metabolic pathways, including solvent production, is still not fully described in clostridia.

Spore or pre-spore formation was surprisingly not observed during cultivation of the OESpo0A mutant strain. This shows that high levels of expression of *spo0A* itself is not able to initiate sporulation in *C. beijerinckii* NRRL B-598. There is the question of why *spo0A* overexpression did not launch expression of other genes in the sporulation cascade. This could be due to the corresponding protein *spo0A* not being formed or its formation not corresponding with *spo0A* overexpression because of interactions of small RNAs with corresponding mRNAs. Phosphorylation, which is necessary for protein activation, may also have been impaired. Regulation of expression by small RNAs was predicted in *C. acetobutylicum* and *C. beijerinckii* (Chen et al. 2011) and was proven in *C. acetobutylicum* (Venkataramanan et al. 2013). In contrast, good agreement of *spo0A* expression with relevant protein formation in *C.acetobutylicum* ATCC 824 was confirmed by Sullivan and Bennett (2006).

This behaviour of the OESpo0A strain is generally in contrast with expectations and previous findings for *C. acetobutylicum* in high levels of *spo0A* expression led to higher solvent production compared to the control strain and produced only ca. 25% less butanol than the WT (Harris et al. 2002). Overexpression of the *spo0A* gene in *C. acetobutylicum* also significantly accelerated sporulation compared to the WT. However, in a different study, overexpression of *spo0A* in a mutant of *C. acetobutylicum* could not be completely correlated with high butanol production and butanol production was found to be the highest under moderate expression of *spo0A* (Alsaker et al. 2004).

The interconnection between sporulation and solvent production is an often discussed question in ABE fermentation, and opinions regarding these issues have changed over the last few years. *C. acetobutylicum* ATCC824 and *C. beijerinckii* NCIMB 8052 mutants with a disrupted *spo0A* (Harris et al. 2002; Ravagnani et al. 2000) abolished granulose and spore formation, and exhibited a significant reduction in solvent production. A *C. acetobutylicum* mutant with Spo0A overexpression (Harris et al. 2002) was able to produce more spores and solvents in comparison with the control strain bearing a parental plasmid but not compared to the WT. A possible link between expression of solventogenic genes and activity of Spo0A was also described previously in several high-throughput transcriptomic studies (Tomas et al. 2004; Wang et al. 2012). On the contrary, Grimmler et al. (2011) demonstrated independent transcriptional regulation of sporulation and solventogenesis, at least during phosphate-limited continuous cultivation. More recently, it was found that *C. pasteurianum* strain M150B bearing a deletion in the *spo0A* gene could not produce granulose but produced significantly more butanol and exhibited a shorter lag phase (Sandoval et al. 2015). Furthermore, *C. beijerinckii* NCIMB 8052 *spo0A* deficient strain prepared by the new CRISPR/Cas methodology in 2017 (Seo et al. 2017) was able to produce more solvent than a previous mutant prepared using an integrated plasmid (Ravagnani et al. 2000).

Slight overexpression of *ald* and *buk1,* which were observed in OESpo0A in RCM medium, were probably related to high levels of expression of *spo0A,* which may have played a significant role in their regulation, as was described previously (Ravagnani et al. 2000). Higher expression of *ald* and *buk1* did not lead to higher concentrations of butanol or butyric acid in RCM culture. Unfortunately, no mutant strain of *C. beijerinckii* with *ald* overexpression has been described, but a similar situation was observed, e.g. by Nair et al. (1994) and Harris et al. (2000) in the case of *adhE* in *C. acetobutylicum*. There is also an open question of how many alcohol/aldehyde dehydrogenases contribute to the formation of butanol and ethanol in *C. beijerinckii* NRRL B-598 since in *C. acetobutylicum* DSM 1731, multiple enzymes encoded by multiple genes have been found to contribute to alcohol formation (Dai et al. 2016).

Expressions of *spoIIE*, *sigG* and *spoVD* were a little higher in OESpo0A than in control or WT strains but still lower than in TYA culture of the WT. These results were contrary to observations in *C. acetobutylicum* bearing *spo0A* overexpression, where expressions of *sigG*, *buk1*

and *adhE* were observed earlier compared to the control strain (Harris et al. 2002). On the other hand, Alsaker et al. (2004) showed in the same mutant strain that *spo0A* overexpression led to downregulation of many glycolytic genes, which could explain poor growth of the mutant compared to WT. The position of a mutated 0A box (TTTGGAA) between promoter and TSS would suggest negative regulation of *SpoIIE* by Spo0A and may explain unexpected down regulation of *spoIIE*. The *spoIIE* sequence of *C. acetobutylicum* ATCC 824 that was proven to be positively regulated by Spo0A (Paredes et al. 2004) shares only 59% homology with *C. beijerinckii* strains, is carried on the antisense strand, and contains three putative 0A boxes (TGACAAA, CATCCAA, TATCAAA) upstream of the promoter. Regarding potential *sigG* regulation by Spo0A, a putative A0 box (TGACAAA) is located only 11 bp upstream of the *SpoIIG* operon, also suggesting negative regulation by Spo0A. In addition, a lower binding affinity and mutated binding sites of the *spoIIG* promoter for Spo0A was proven in *B. subtilis* (Fujita et al. 2005). Mutated 0A boxes upstream of the sol operon promoter suggest positive regulation of *ald* by Spo0A and explain higher expression of *ald* in strain OESpo0A.

From the point of view of industrial solvent production, an asporogenous production strain would be advantageous because sporulation is an energy demanding process and part of the carbon source is lost for sporulation and therefore not used in the synthesis of valuable products. In addition, mature spores are metabolically inactive. Both properties result in decreased yields and productivity in a sporulating culture compared to a non-sporulating one. However, an asporogenous and simultaneous solvent-forming clostridial phenotype was observed only rarely, usually during continuous cultivation of *C. acetobutylicum,* strains DSM1791 and ATCC 824 (Meinecke et al. 1984; Grimmler et al. 2011). A model displaying the importance of non-sporulating cultures for butanol production was described earlier by Qureshi et al. (1988). These authors showed unequal contributions to product (butanol) formation by different sub-populations in a usually heterogeneous sporulating clostridial culture. In *C. beijerinckii* NRRL B-598, the non-sporulating but butanol-producing phenotype has been described under specific cultivation conditions (Kolek et al. 2016a) and the results obtained in this study have confirmed this observation.

C. beijerinckii NRRL B-598 is able to form butanol in sporulating as well as non-sporulating cultures, suggesting that solventogenesis is not tightly related to sporulation in this strain. Surprisingly it was found that the gene for the main sporulation regulator, Spo0A, was overexpressed while other monitored sporulation factors, including sigma factor G were downregulated in a non-sporulating culture. Similarly, surprising results were also obtained in an OESpo0A strain that did not produce spores and *spo0A* overexpression led to downregulation of other monitored sporulation factors; this was contrary to expectations.

These results support the theory that regulation of solventogenesis by Spo0A is not the same in all solventogenic clostridia, as previously proven by Seo et al. (2017) and Sandoval et al. (2015), although the solventogenic species are genetically closely related to each other. It would be beneficial to study respective protein formation and their potential activation (phosphorylation) to be able to elucidate the effect of *spo0A* overexpression. Activated Spo0A probably induces production of *ald* and other genes of the solventogenic pathway. However, the level of Spo0A transcripts does not always fit with butanol production, as has been shown for a mutant of *C. acetobutylicum* ATCC 824 (Alsaker et al. 2004), which is an example of the diversity in mechanisms regulating sporulation and solvent production in clostridia.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human participants or animals performed by any of the authors.

References

Al-Hinai MA, Jones SW, Papoutsakis (2015) The *Clostridium* sporulation programs: diversity and preservation of endospore differentiation. Microbiol Mol Biol Rev. https://doi.org/10.1128/MMBR.00025-14

Alsaker KV, Papoutsakis (2005) Transcriptional program of early sporulation and stationary-phase events in *Clostridium acetobutylicum*. J Bacteriol. https://doi.org/10.1128/JB.187.20.7103-7118.2005

Alsaker KV, Spitzer TR, Papoutsakis (2004) Transcriptional analysis of spo0A overexpression in *Clostridium acetobutylicum* and its effect on the cell's response to butanol stress. J Bacteriol. https://doi.org/10.1128/JB.186.7.1959-1971.2004

Bahl H, Andersch W, Gottschalk G (1982) Continuous production of acetone and butanol by *Clostridium acetobutylicum* in a

two-stage phosphate limited chemostat. Eur J Appl Microbiol Biotechnol 15:201–205

Brown DP, Ganova-Raeva L, Green BD, Wilkinson SR, Young M, Youngman P (1994) Characterization of *spo0A* homologues in diverse *Bacillus* and *Clostridium* species identifies a probable DNA-binding domain. Mol Microbiol. https://doi.org/10.1111/j.1365-2958.1994.tb02176.x

Chen Y, Indurthi DC, Jones SW, Papoutsakis (2011) Small RNAs in the genus *Clostridium*. MBio. https://doi.org/10.1128/mBio.00340-10

Dai Z, Dong H, Zhang Y, Li Y (2016) Elucidating the contributions of multiple aldehyde/alcohol dehydrogenases to butanol and ethanol production in *Clostridium acetobutylicum*. Sci Rep. https://doi.org/10.1038/srep28189

Fujita M, González-Pastor JE, Losick R (2005) High- and low-threshold genes in the Spo0A regulon of *Bacillus subtilis*. J Bacteriol. https://doi.org/10.1128/JB.187.4.1357-1368.2005

Grimmler C, Janssen H, Krausse D, Fischer RJ, Bahl H, Durre P, Liebl W, Ehrenreich A (2011) Genome-wide gene expression analysis of the switch between acidogenesis and solventogenesis in continuous cultures of *Clostridium acetobutylicum*. J Mol Microbiol Biotechnol. https://doi.org/10.1159/000320973

Harris LM, Desai RP, Welker NE, Papoutsakis (2000) Characterization of recombinant strains of the *Clostridium acetobutylicum* butyrate kinase inactivation mutant: need for new phenomenological models for solventogenesis and butanol inhibition? Biotechnol Bioeng. https://doi.org/10.1002/(SICI)1097-0290(20000105)67:1<1::AID-BIT1>3.0.CO;2-G

Harris LM, Welker NE, Papoutsakis (2002) Northern, morphological, and fermentation analysis of spo0A inactivation and overexpression in *Clostridium acetobutylicum* ATCC 824. J Bacteriol. https://doi.org/10.1128/JB.184.13.3586-3597.2002

Hatt JK, Youngman P (2000) Mutational analysis of conserved residues in the putative DNA-binding domain of the response regulator Spo0A of *Bacillus subtilis*. J Bacteriol. https://doi.org/10.1128/JB.182.24.6975-6982.2000

Heap JT, Pennington OJ, Cartman ST, Minton NP (2009) A modular system for *Clostridium* shuttle plasmids. J Microbiol Methods. https://doi.org/10.1016/j.mimet.2009.05.004

Higgins D, Dworkin J (2012) Recent progress in *Bacillus subtilis* sporulation. FEMS Microbiol Rev. https://doi.org/10.1111/j.1574-6976.2011.00310.x

Huang IH, Waters M, Grau RR, Sarker MR (2004) Disruption of the gene (*spo0A*) encoding sporulation transcription factor blocks endospore formation and enterotoxin production in enterotoxigenic *Clostridium perfringens* type A. FEMS Microbiol Lett. https://doi.org/10.1016/j.femsle.2004.02.014

Jones DT, Woods DR (1986) Acetone-butanol fermentation revisited. Microbiol Rev 50:484–524

Jones SW, Paredes CJ, Tracy B, Cheng N, Sillers R, Senger RS, Papoutsakis (2008) The transcriptional program underlying the physiology of clostridial sporulation. Genome Biol. https://doi.org/10.1186/gb-2008-9-7-r114

King N, Dreesen O, Straiger P, Pogliano K, Losick R (1999) Septation, dephosphorylation, and the activation of sigmaF during sporulation in *Bacillus subtilis*. Genes Dev 13:1156–1167

Klucar L, Stano M, Hajduk M (2010) phiSITE: database of gene regulation in bacteriophages. Nucleic Acids Res. https://doi.org/10.1093/nar/qkp911

Kolek J, Branska B, Drahokoupil M, Patakova P, Melzoch K (2016a) Evaluation of viability, metabolic activity and spore quantity in clostridial cultures during ABE fermentation. FEMS Microbiol Lett. https://doi.org/10.1093/femsle/fnw031

Kolek J, Sedlar K, Provaznik I, Patakova P (2016b) Dam and Dcm methylations prevent gene transfer into *Clostridium pasteurianum* NRRL B-598: development of methods for electrotransformation, conjugation, and sonoporation. Biotechnol Biofuels. https://doi.org/10.1186/s13068-016-0436-y

Lee KY, Juang TC, Lee KC (1978) Effect of metal ions on growth and sporulation of *Clostridium perfringens* in a synthetic medium. Zhonghua Min Guo Wei Sheng Wu Xue Za Zhi 11:50–61

Lipovsky J, Patakova P, Paulova L, Pokorny T, Rychtera M, Melzoch K (2016) Butanol production by *Clostridium pasteurianum* NRRL B-598 in continuous culture compared to batch and fed-batch systems. Fuel Process Tech. https://doi.org/10.1016/j.fuproc.2015.12.020

Long S, Jones DT, Woods DR (1983) Sporulation of *Clostridium acetobutylicum* P262 in a defined medium. Appl Environ Microbiol 45:1389–1393

Mearls EB, Lynd LR (2014) The identification of four histidine kinases that influence sporulation in *Clostridium thermocellum*. Anaerobe. https://doi.org/10.1016/j.anaerobe.2014.06.004

Meinecke B, Bahl H, Gottschalk G (1984) Selection of an asporogenous strain of *Clostridium acetobutylicum* in continuous culture under phosphate limitation. Appl Environ Microbiol 48:1064–1065

Nair RV, Bennett GN, Papoutsakis ET (1994) Molecular characterization of an aldehyde/alcohol dehydrogenase gene from *Clostridium acetobutylicum* ATCC 824. J Bacteriol 176: 871–85

Paredes CJ, Rigoutsos I, Papoutsakis (2004) Transcriptional organization of the *Clostridium acetobutylicum* genome. Nucleic Acids Res. https://doi.org/10.1093/nar/gkh509

Piggot PJ, Hilbert DW (2004) Sporulation of *Bacillus subtilis*. Curr Opin Microbiol. https://doi.org/10.1016/j.mib.2004.10.001

Qureshi N, Paterson AHJ, Maddox IS (1988) Model for continuous production of solvents from whey permeate in a packed bed reactor using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar. Appl Microbiol Biotechnol 29:323–328

Ravagnani A, Jennert KC, Steiner E, Grunberg R, Jefferies JR, Wilkinson SR, Young DI, Tidswell EC, Brown DP, Youngman P, Morris JG, Young M (2000) Spo0A directly controls the switch from acid to solvent production in solvent-forming clostridia. Mol Microbiol. https://doi.org/10.1046/j.1365-2958.2000.02071.x

Robson RL, Robson RM, Morris JG (1974) The biosynthesis of granulose by *Clostridium pasteurianum*. Biochem J 144:503–511

Sandoval NR, Venkataramanan KP, Groth TS, Papoutsakis (2015) Whole-genome sequence of an evolved *Clostridium pasteurianum* strain reveals Spo0A deficiency responsible for increased butanol production and superior growth. Biotechnol Biofuels. https://doi.org/10.1186/s13068-015-0408-7

Sauer M (2016) Industrial production of acetone and butanol by fermentation-100 years later. FEMS Microbiol Lett. https://doi.org/10.1093/femsle/fnw134

Sedlar K, Kolek J, Skutkova H, Branska B, Provaznik I, Patakova P (2015) Complete genome sequence of *Clostridium pasteurianum* NRRL B-598, a non-type strain producing butanol. J Biotechnol. https://doi.org/10.1016/j.jbiotec.2015.09.022

Sedlar K, Kolek J, Provaznik I, Patakova P (2017) Reclassification of non-type strain *Clostridium pasteurianum* NRRL B-598 as *Clostridium beijerinckii* NRRL B-598. J Biotechnol. https://doi.org/10.1016/j.jbiotec.2017.01.003

Seo SO, Wang Y, Lu T, Jin YS, Blaschek HP (2017) Characterization of a *Clostridium beijerinckii spo0A* mutant and its application for butyl butyrate production. Biotechnol Bioeng. https://doi.org/10.1002/bit.26057

Steiner E, Dago AE, Young DI, Heap JT, Minton NP, Hoch JA, Young M (2011) Multiple orphan histidine kinases interact directly with Spo0A to control the initiation of endospore formation in *Clostridium acetobutylicum*. Mol Microbiol. https://doi.org/10.1111/j.1365-2958.2011.07608.x

Sullivan L, Bennett GN (2006) Proteome analysis and comparison of *Clostridium acetobutylicum* ATCC 824 and Spo0A strain variants.

J Ind Microbiol Biotechnol. https://doi.org/10.1007/s10295-005-0050-7

Tomas CA, Beamish J, Papoutsakis (2004) Transcriptional analysis of butanol stress and tolerance in *Clostridium acetobutylicum*. J Bacteriol. https://doi.org/10.1128/JB.186.7.2006-2018.2004

Venkataramanan KP, Jones SW, McCormick KP, Kunjeti SG, Ralston MT, Meyers BC, Papoutsakis (2013) The *Clostridium* small RNome that responds to stress: the paradigm and importance of toxic metabolite stress in *C. acetobutylicum*. BMC Genomics. https://doi.org/10.1186/1471-2164-14-849

Wang Y, Li X, Mao Y, Blaschek HP (2012) Genome-wide dynamic transcriptional profiling in *Clostridium beijerinckii* NCIMB 8052 using single-nucleotide resolution RNA-Seq. BMC Genomics. https://doi.org/10.1186/1471-2164-13-102

Yang J, Chen X, McDermaid A, Ma Q (2017) DMINDA 2.0:integrated and systematic views of regulatory DNA motif identification and analyses. Bioinformatics. https://doi.org/10.1093/bioinformatics/btx223

York K, Kenney TJ, Satola S, Moran CP Jr, Poth H, Youngman P (1992) Spo0A controls the σ^A–dependent activation of *Bacillus subtilis* sporulation-specific transcription unit *spoIIE*. J Bacteriol 174: 2648–2658