

SCIENTIFIC REPORTS



OPEN

σ^{54} (σ^L) plays a central role in carbon metabolism in the industrially relevant *Clostridium beijerinckii*

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The solventogenic *C. beijerinckii* DSM 6423, a microorganism that naturally produces isopropanol and butanol, was previously modified by random mutagenesis. In this work, one of the resulting mutants was characterized. This strain, selected with allyl alcohol and designated as the AA mutant, shows a dominant production of acids, a severely diminished butanol synthesis capacity, and produces acetone instead of isopropanol. Interestingly, this solvent-deficient strain was also found to have a limited consumption of two carbohydrates and to be still able to form spores, highlighting its particular phenotype. Sequencing of the AA mutant revealed point mutations in several genes including CIBE_0767 (*sigL*), which encodes the σ^{54} sigma factor. Complementation with wild-type *sigL* fully restored solvent production and sugar assimilation and RT-qPCR analyses revealed its transcriptional control of several genes related to solventogenesis, demonstrating the central role of σ^{54} in *C. beijerinckii* DSM 6423. Comparative genomics analysis suggested that this function is conserved at the species level, and this hypothesis was further confirmed through the deletion of *sigL* in the model strain *C. beijerinckii* NCIMB 8052.

In the context of worldwide energy transition, research for alternatives to fossil fuels has become a priority. In particular, the replacement of petrochemistry by a low carbon emission industry has been a major challenge as our global consumption of petrochemicals keeps on increasing¹. The valorization of plant biomass to synthesize ethanol by microbial fermentation has already been pioneered for biofuel production² and could therefore be applied to bio-based chemistry³.

A few strains from the *Clostridium* genus are naturally able to produce isopropanol and butanol^{4,5}, two compounds that could be used as biochemical and biofuel, respectively. However, those organisms are not producing these metabolites in quantities compatible with an economically viable industrial process⁶. However, with the increasing availability of efficient genetic tools in *Clostridia*⁷, metabolic engineering approaches could be undertaken to enhance solvent productivity. *Clostridium beijerinckii* DSM 6423 (NRRL B-593) is the only natural isopropanol-butanol producing strain whose genome and transcriptome have been investigated⁸. It may therefore be the best candidate for genetic engineering, although its particular physiology is still poorly understood. For this purpose, gaining additional knowledge on metabolism regulation in this strain would greatly benefit future optimization efforts. In particular, identifying the molecular effectors controlling solvent production may provide valuable insights to define adequate genetic engineering strategies.

As no genetic toolbox was available for this particular strain, Máté de Gerando and coworkers performed random mutagenesis coupled with genome shuffling to increase isopropanol productivity by selecting isopropanol-tolerant strains⁹. In this work, random mutagenesis followed by allyl alcohol selection also generated an interesting mutant, further referred to as AA mutant. This strain mainly produces acids, shows no isopropanol production and a strongly attenuated butanol synthesis capacity. These results are consistent with those obtained in *Clostridium acetobutylicum* DSM 1792, in which mutants obtained in the presence of allyl alcohol - precursor of the highly toxic acrolein molecule in the reaction catalyzed by alcohol dehydrogenases - permitted the selection of butanol-deficient strains¹⁰. Nevertheless, in both cases the key mutated genes causing these phenotypes have not been clearly identified.

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In this study, we demonstrate that the solvent production deficiency in the AA mutant is due to a point mutation in the CIBE_0767 (*sigL*) gene, which encodes the transcriptional regulator σ^{54} (also referred to as σ^L). Similarly to other sigma factors, σ^{54} regulates genetic expression by incorporating the RNA polymerase complex and binding to specific promoter sequences, thus enabling selective transcription of a subset of genes^{11–13}. The importance of this sigma factor for solventogenesis was further confirmed at the species level by deleting the corresponding gene in the *C. beijerinckii* model strain (i.e. NCIMB 8052). In addition to regulating alcohol synthesis pathways, our experiments revealed that this sigma factor also controls utilization of alternative carbon sources, such as lactose and cellobiose, and is not required to complete the sporulation process, making it a central and specific controller of carbon metabolism in *C. beijerinckii*.

Results

The *C. beijerinckii* AA mutant metabolism is orientated towards acid production. Phenotypic characterization of a solventogenic *Clostridium* is usually assessed by the identification of fermentation products. We therefore performed triplicate fermentation assays of both *C. beijerinckii* DSM 6423 wild-type and AA strains to compare their product pattern after 48 hours in Gapes medium.

When compared to the wild-type strain, the AA mutant showed a very distinctive behavior. Firstly, growth was strongly impacted with a ca. 2-fold decrease in OD₆₀₀ units after 48 hours of fermentation in Gapes medium, influencing end-products concentrations and glucose consumption (Fig. 1, Supplementary File S1).

Although butanol was still produced, the central metabolism of the AA strain shifted towards acid formation, with notably a strong increase in butyrate concentration resulting in a final solvent:acid ratio of 1:3, compared to 6:1 in the wild-type strain (Fig. 1c).

The phenotype of the AA mutant, mainly producing butyrate, may result from the coupled inactivation of one or several gene(s) involved in isopropanol and butanol production.

The AA mutant is still able to complete the sporulation process. Complete or partial loss of solventogenesis in the *C. beijerinckii* species is often associated with a degeneration phenomenon (i.e. loss of the capacity to form spores in addition to the loss of solvent production)¹⁴. Given its phenotype, we therefore decided to investigate the sporulation ability of the AA mutant.

A comparative assay confirmed that sporulation ability was conserved, despite an approximate 10/100-fold decrease in the number of spores in comparison to the wild-type strain (Supplementary File S1). However, given the limited growth of the AA strain, this decrease may not necessarily be linked to a genetic deficiency but could instead result from a diminished ability to accumulate population density.

Importantly, demonstration of the sporulation ability in the AA strain makes its phenotype unrelated to the degeneration phenomenon, which suggests that its genome contains solventogenic-specific mutations.

A point mutation in the *sigL* gene is predicted to impede solvent formation and sugar consumption. We next adopted a forward genetic approach to identify the gene(s) responsible for the AA phenotype. Whole-genome sequencing followed by read mapping on the wild-type genome allowed the identification of 13 SNPs (Table 1) along the chromosome of the strain. Among the affected genes, CIBE_0767 particularly retained our attention as P-BLAST analysis against Swissprot database revealed high similarities with putative RNA polymerase σ^{54} factors (encoded by the *sigL* gene) and notably the one from *Clostridium kluyveri*¹⁵. In the AA strain, the SNP detected in the *sigL* gene causes a serine to phenylalanine substitution at position 366. The σ^{54} transcriptional factor has already been well described in several bacterial species and contains three major domains¹² (Fig. 2a). Region I (RI) is involved in core RNA polymerase, enhancer, and DNA binding. Region II (RII) is acidic and poorly conserved. Region III (RIII) is divided in several conserved sub-regions interacting with core RNA polymerase (CBD) and DNA at the -12 consensus motif (HTH) and the -24 motif (RpoN). *In silico* analysis of the *sigL* gene revealed that the amino acid substitution (S366F) is localized in the HTH motif, at an extremely conserved position (Fig. 2b). Given the significant difference in terms of steric hindrance, polarity and hydrophobicity resulting from a phenylalanine to serine substitution, this mutation may drastically impede the -12-motif recognition, yielding a partial or total inactivation of σ^{54} .

We subsequently looked at the predicted σ^{54} -regulon along the chromosome of *C. beijerinckii* DSM 6423. The σ^{54} binding motif consensus (TGGCANNNNNTTGCW), based on the study of validated σ^{54} promoters by Barrios and colleagues¹³, was found at 57 genomic sites. Among them, 35 were localized up to a few hundred base pairs upstream of a coding sequence (Supplementary File S2). In particular, two genes coding for strongly expressed⁸ alcohol dehydrogenases (CIBE_2050 and CIBE_2622, which convert acetaldehyde and butyraldehyde into ethanol and butanol, respectively)^{16,17} as well as the secondary alcohol dehydrogenase s-ADH encoding gene (CIBE_3470, whose product converts acetone to isopropanol)^{16,18,19} were identified (Fig. 2c). Although 2,3-butanediol is usually not detected in our culture conditions, the only gene predicted to encode a 2,3-butanediol dehydrogenase (CIBE_1696, similar to the one described by Raedts *et al.*)²⁰ was identified as being part of the regulon.

Unlike other sigma factors, σ^{54} -bound RNA polymerase requires the ATPase activity of an adjacent Enhancer Binding Protein (EBP) to start transcription initiation^{21,22}. Importantly, genes encoding σ^{54} EBPs were found in the direct vicinity of CIBE_2622, CIBE_3470 and CIBE_1696, which strengthens the hypothesis of a σ^{54} -driven transcriptional control of their expression.

In order to investigate the functionality of the σ^{54} -regulon, we used the synteny tool from MaGe platform (16) to compare the position of σ^{54} binding sites in *C. beijerinckii* DSM 6423 with two other *C. beijerinckii* model strains (*C. beijerinckii* NCIMB 8052 and the recently reannotated NRRL B-598)^{23,24}. Apart from the s-ADH encoding gene which drives isopropanol production and is only present in the DSM 6423 strain, these putative σ^{54} -dependent transcriptional units appeared to be conserved in these acetone-butanol producing strains.

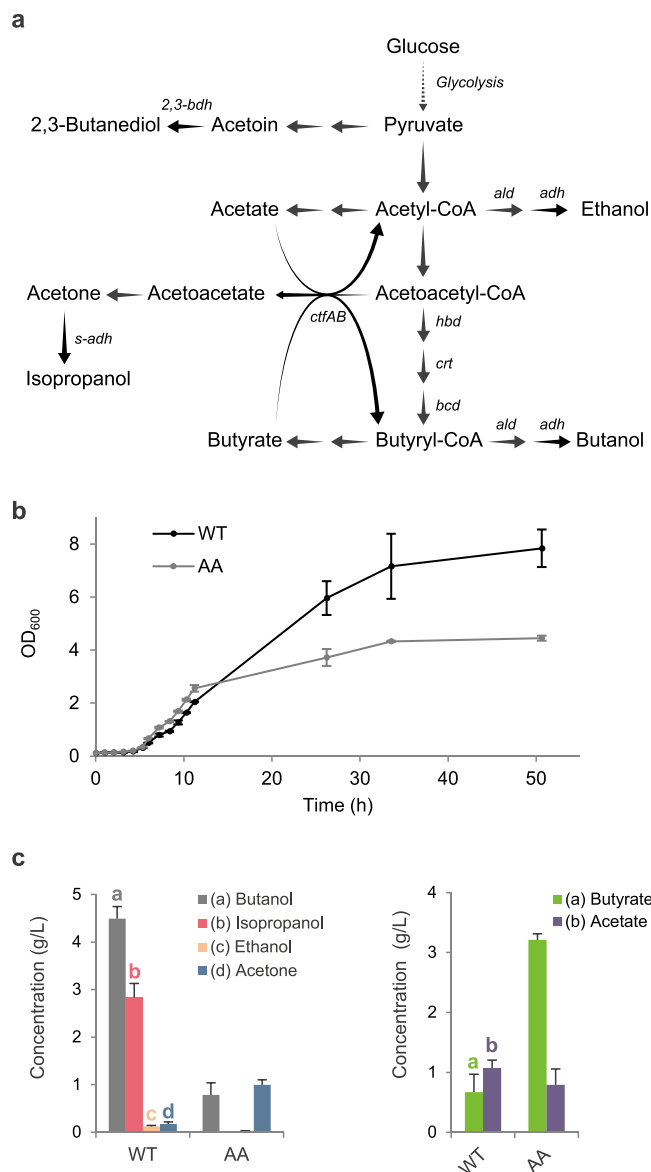


Figure 1. Comparative phenotypic analysis of *C. beijerinckii* DSM 6423 wild-type and AA strains. (a) Simplified central metabolism of *C. beijerinckii* DSM 6423. (b) Growth kinetics comparison between *C. beijerinckii* DSM 6423 (WT) and the AA mutant. (c) Final solvent and acid concentrations measured after 48 h of fermentation in Gapes medium for wild-type *C. beijerinckii* DSM 6423 and the AA mutant. Error bars indicate the standard deviation of triplicate experiments.

Besides, downregulation of the butanol and secondary alcohol dehydrogenases linked to σ^{54} potential inactivation would match the AA phenotype, which gives another hint that *sigL* mutation may be significant.

Interestingly, other genes predicted to be part of the σ^{54} -regulon are mainly associated with sugar uptake (mainly PTS-based transporters, for a review in solventogenic *Clostridia*, see Mitchell, 2015)²⁵ and metabolism (Fig. 2d, Supplementary File S2). Lactose and cellobiose transport and metabolism are also predicted to be regulated by σ^{54} . These operons contain genes encoding a phosphotransferase system (PTS) for sugar transport and a hydrolase (β -galactosidase CIBE_0885; β -glucosidase CIBE_1708). Interestingly, a highly expressed and conserved operon encoding a dihydroxyacetone phosphate kinase complex (*dhaKLM*; CIBE_2581-2582-2583) also appears to be controlled by σ^{54} . This enzyme can be involved in glycerol metabolism, or directly in the central carbon pathway when associated to 6P-fructose aldolases^{26,27}. Notably, two 6P-fructose aldolases (CIBE_0334 and CIBE_0411), one of which being well expressed in the available RNA-seq dataset⁸, are annotated in the DSM 6423 genome. Lastly, parts of the pentose phosphate pathway also seem to be regulated by σ^{54} , with two conserved similar operons encoding transketolases, ribose/ribulose phosphate epimerases and isomerases (Supplementary File S2). Importantly, some of these putative σ^{54} -dependent transcriptional units appeared to be well conserved in the NCIMB 8052 and NRRL-B598 strains (Fig. 2d).

Locus tag	Description	Change	Genome position	CDS Position	Amino Acid Change	Protein Effect
CIBE_0366	Conserved protein of unknown function	C -> T	390,092	2,215	Q -> X	Truncation
CIBE_0767	Putative RNA polymerase sigma-54 factor	C -> T	770,679	1,097	S -> F	Substitution
CIBE_1279	Conserved exported protein of unknown function	C -> T	1,347,126	31	none	None
CIBE_1303	Conserved protein of unknown function	A -> G	1,373,758	218	Y -> C	Substitution
N/A ^a	Intergenic	G -> A	1,691,364	N/A ^a	N/A ^a	N/A ^a
CIBE_2357	Binding-protein-dependent transport systems inner membrane component	G -> A	2,397,512	614	G -> D	Substitution
CIBE_2358	NMT1/THI5-like protein	G -> A	2,397,959	138	None	None
CIBE_2486	Protein of unknown function	C -> T	2,535,980	103	P -> S	Substitution
CIBE_2488	Protein of unknown function	C -> T	2,537,804	157	P -> S	Substitution
CIBE_3408	Putative hydrolase subunit antagonist of KipI	C -> T	3,476,677	206	T -> I	Substitution
CIBE_3475	Histidine kinase	C -> A	3,572,491	732	R -> S	Substitution
CIBE_4586	Dihydroxy-acid dehydratase	G -> T	4,675,905	1,159	V -> F	Substitution
N/A ^a	Intergenic	C -> T	4,931,169	N/A ^a	N/A ^a	N/A ^a
CIBE_5453	Short chain acyl-CoA transferase: fused alpha subunit; beta subunit	A -> G	5,622,646	919	S -> P	Substitution
CIBE_5584	Putative ABC transporter (ATP-binding protein)	G -> A	5,774,626	146	R -> K	Substitution

Table 1. Mutations detected in the AA strain. ^aNot applicable.

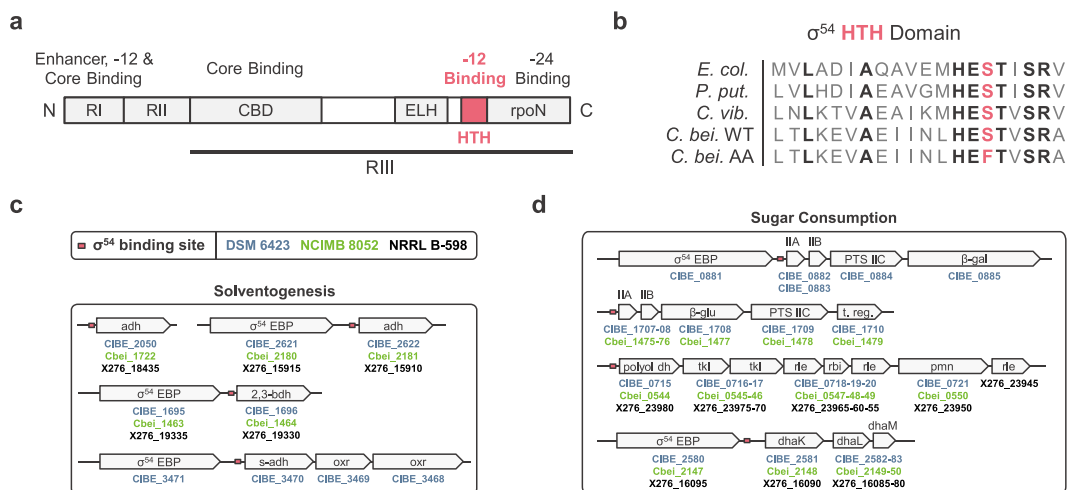


Figure 2. Structural position of σ^{54} mutation in *C. beijerinckii* DSM 6423 AA strain and predictions for this sigma factor role in several model strains. **(a)** Structural organization of the σ^{54} sigma factor. RI-III: region I to III. CBD: core binding domain. ELH: extra-long α -helix. HTH: helix turn helix motif. rpoN: RNA polymerase factor N domain. **(b)** Localization of the S336F mutation identified in the AA strain. *E. coli.*: *Escherichia coli*; *P. put.*: *Pseudomonas putida*; *C. vib.*: *Caulobacter vibrioides*; *C. bei.*: *Clostridium beijerinckii*. **(c,d)** In silico predictions of **(c)** solvent production and **(d)** sugar metabolism regulation by σ^{54} in several *C. beijerinckii* strains. Transcriptional units involved in these pathway bear an upstream σ^{54} binding site (consensus: TGGCANNNNNTTGCW). *adh*: alcohol dehydrogenase; *2,3-bdh*: 2,3-butanediol dehydrogenase; *s-adh*: secondary alcohol dehydrogenase; *oxr*: oxidoreductase. PTS: phosphotransferase system; β -gal: β -galactosidase; β -glu: β -glucosidase; *t. reg.*: transcriptional regulator; *dhaK/L/M*: dihydroxyacetone kinase subunits K/L/M; *polyol dh*: polyol dehydrogenase; *tkl*: transketolase; *rle*: ribulose phosphate epimerase; *rbi*: ribose phosphate isomerase; *pmm*: phosphomannomutase.

In summary, analysis of the AA genotype revealed an amino acid substitution potentially inactivating a protein predicted not only to regulate solventogenesis but also other central metabolic pathways such as sugar consumption in several *C. beijerinckii* strains.

Complementation with wild-type *sigL* gene fully restores solventogenesis and sugar metabolism in the AA strain. As several SNPs were detected in the genome of the AA mutant, complementation assays were required to characterize the corresponding phenotype. Considering the predicted crucial role of the putative σ^{54} -encoding gene (*sigL*, CIBE_0767), we decided to clone the wild-type coding sequence of *sigL* under the control of its endogenous promoter in the pFW01 vector, yielding the pFW- σ^{54} plasmid, and to introduce

it into both wild-type and AA strains. Fermentation analysis revealed that both WT and AA pFW- σ^{54} strains displayed a wild-type solventogenic metabolism (Fig. 3). Indeed, normal acid and solvent production (Fig. 3a), as well as glucose consumption and biomass production (Supplementary File S3) were fully restored in the AA pFW- σ^{54} strain when compared to the wild-type strain containing the empty vector. Interestingly, the WT pFW- σ^{54} recombinant strain did not exhibit any major change when compared to the wild-type microorganism.

These results confirmed that CIBE_0767, encoding the σ^{54} transcriptional factor, is drastically influencing the regulation of the central metabolism of the AA strain in our conditions. We therefore kept on investigating its impact on other predicted metabolic pathways.

Plate assays on minimal medium were used to analyze carbon sources usage. In our conditions, no effect after 24 or 48 hours was seen on bacterial growth on fructose, mannose, xylose, arabinose, sucrose, glycerol, inulin, starch or mannitol (data not shown). In contrast, growth was impeded with cellobiose and lactose in the AA control strain (empty vector), and restored when complemented with wild-type σ^{54} (Fig. 3b). However, quantification of this inhibition in liquid medium revealed unexpected results as growth was not altered on 2YT 20 g/L cellobiose medium (data not shown) whereas lactose did exert a strong effect on bacterial growth. After 24 h of fermentation the AA pFW01 strain had grown similarly on 2YT 20 g/L lactose than on the 2YT control condition containing no supplementary carbon source (Fig. 3c). HPLC analysis confirmed that almost no lactose was consumed by the AA strain in this time frame. On the other hand, normal growth on lactose was observed with the complementation strain with a consumption of ca. 8 g/L of lactose in 24 h. The fermentation was carried out for 72 more hours, and end-point analysis revealed that lactose uptake inhibition was only partial (Supplementary File S4).

Transcriptional control of the predicted σ^{54} regulon was further assessed by performing RT-qPCR on the WT/AA strains, with and without *sigL* complementation (Fig. 3d). Expression of CIBE_2050 (*adh-1*), CIBE_2622 (*adh-2*) and CIBE_3470 (*s-adh*) was decreased (c.a. 2.5–5 fold) in the AA strain and complementation resulted in a drastic increase of their expression (≈ 10 times), congruent with the strong expression of *sigL* on a multi-copy plasmid. This stronger expression however did not result in a higher titer of solvents as outlined before. Alcohol synthesis may thus be limited by another factor (e.g. butanol tolerance or another rate-limiting step in the solventogenic pathways) in *C. beijerinckii* DSM 6423.

The *sigL* expression level was interestingly much lower in the WT strain than in the AA mutant, which could be linked to a potential feedback regulation.

As expected, expression of the predicted σ^{54} -independent *ald* gene (CIBE_4606, first gene of the *sol* operon)^{8,28} did not vary among these strains. Similarly, expression of two σ^{54} EBP (CIBE_2621, CIBE_3471) was not affected, indicating that their expression is not driven by σ^{54} . This result was also anticipated, since these genes are not expected to be regulated by σ^{54} .

Altogether, these results indicate that the S366F substitution in the σ^{54} is likely the unique modification in the AA strain that impacts solvent production and sugar assimilation. Moreover, our results suggest that σ^{54} is a master regulator of these metabolic pathways in *C. beijerinckii* DSM 6423.

Overexpression of the acid-reassimilation pathway does not restore a wild-type phenotype in the AA strain. The AA phenotype could be linked to two possible mechanisms: a deficient alcohol dehydrogenase activity in the cell and/or a defect in the acid uptake pathway.

To verify whether acid uptake might be impaired in the AA strain, we designed an experiment in which the acid reassimilation pathway would be overexpressed in the AA mutant. In order to do this, a pFW-FC06 plasmid was constructed to overexpress the *s-adh*, *ctfA*, and *ctfB* genes in an operonic structure. This operon was previously used in an overexpression plasmid^{29–31} or integrated in the chromosome³² to convert the model acetone-butanol producing organism *C. acetobutylicum* ATCC 824 into a performant isopropanol-butanol producer.

Introduction of the pFW-FC06 vector slightly increased solvent production and glucose consumption in the AA strain, compared to the introduction of an empty vector (Supplementary File S5). However, even if acetone was fully converted to isopropanol, the recombinant AA strain containing pFW-FC06 still accumulated butyrate at levels comparable to the AA mutant. The latter observation suggests that, consistently with *in silico* predictions, a CtfA/B complex is still functional in the AA mutant (i.e. overexpression of *ctfA/B* does not have much effect on acid levels, similarly to the wild-type strain). The main metabolism roadblock thus probably comes from the aforementioned deficient butanol synthesis pathway. Indeed, in the AA strain, this result would be expected if cyclic conversion of butyryl-CoA to butyrate, and back to butyryl-CoA via the CtfA/B enzyme, was occurring.

In summary, this experiment gives an additional insight on the molecular mechanisms triggering solventogenesis in *C. beijerinckii*: by regulating alcohol dehydrogenase expression, σ^{54} -mediated transcription may be able to modulate the balance between acidogenesis and solventogenesis.

CRISPR/Cas9-mediated deletion of *sigL* in *C. beijerinckii* NCIMB 8052 results in an AA-like phenotype. Complementation with wild-type σ^{54} in the AA strain shed light on *sigL* role in solventogenesis and sugar uptake. However, given the many mutations found along the genome of the AA mutant, we aimed at confirming that the observed phenotype was the unique effect of *sigL* inactivation by deleting it in a wild-type strain.

The model strain *C. beijerinckii* NCIMB 8052 was chosen as a chassis for this modification since, unlike the DSM 6423 strain, markerless genome editing techniques have already been demonstrated to be effective in this microorganism^{33–35}. More particularly, genetic modifications using CRISPR/Cas9-based tools were recently made available to the *Clostridium* community: this technology comes with several advantages (i.e. genome editing overall efficiency, simplicity and rapidity), particularly interesting for genetic engineering of the notably difficult-to-modify clostridial strains, as reviewed recently by Xue *et al.*³⁶.

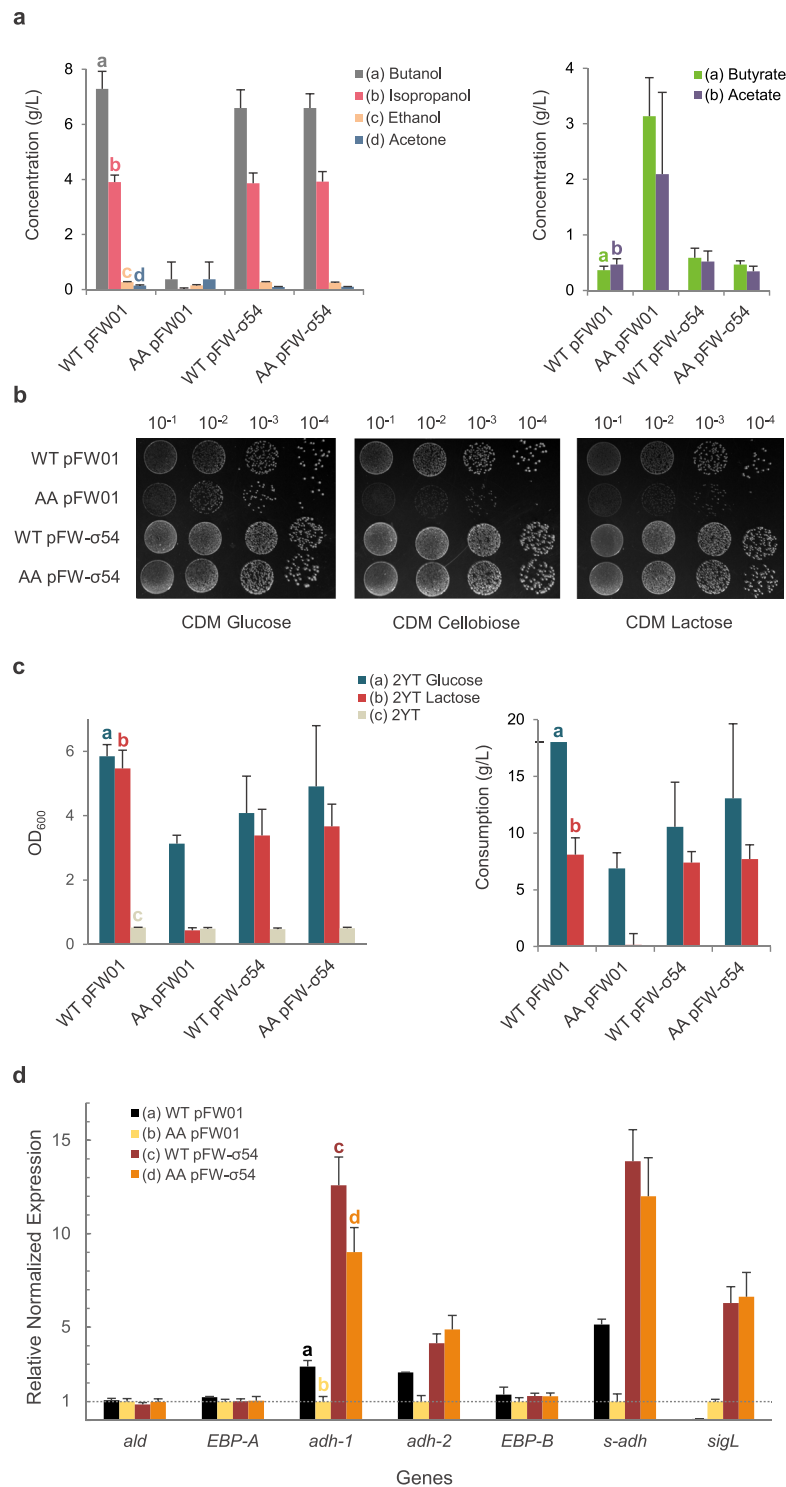


Figure 3. Phenotypic analysis of *C. beijerinckii* DSM 6423 σ^{54} complementation in the AA strain. (a) Fermentation products after 72 hours in Gapes 60 g/L glucose medium of *C. beijerinckii* DSM 6423 wild-type (WT), AA strains containing an empty plasmid (pFW01) or the complementation plasmid (pFW01- σ^{54}). (b) Sugar utilization assay on CDM plates containing 20 g/L glucose, cellobiose or lactose. Pictures were taken after 24 h of incubation on glucose and cellobiose, and 48 h on lactose. (c) Sugar utilization assay in liquid 2YT with 20 g/L glucose (2YTG), lactose (2YTTL) or without added carbon source (2YT). Biomass (given as a measure of optical density at 600 nm, OD₆₀₀) and carbon consumption are measured 24 h after the beginning of the fermentation. (d) RT-qPCR assay measuring the stationary-phase expression of several genes (*ald*: CIBE_4606; *EBP-A*: CIBE_2621; *adh-1*: CIBE_2050; *adh-2*: CIBE_2622; *EBP-B*: CIBE_3471; *s-adh*: CIBE_3470; *sigL*: CIBE_0767). Values are given relative to the AA pFW01 expression value. Error bars indicate the standard deviation of triplicate experiments, except for qPCR data that were obtained in duplicates.

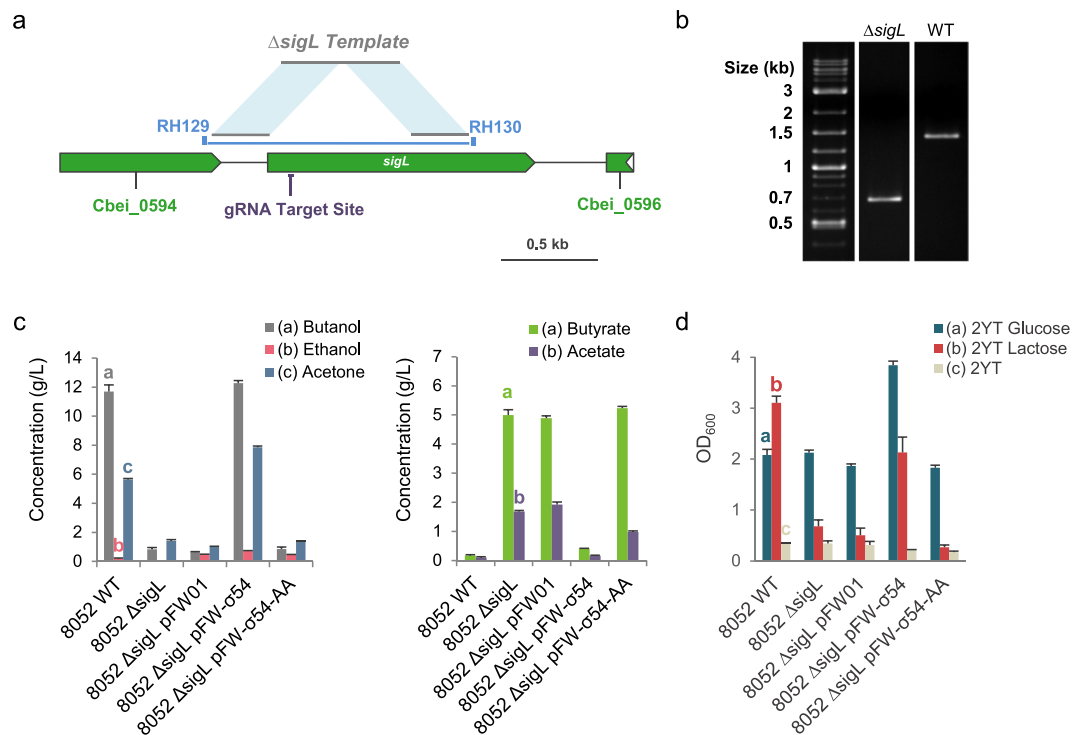


Figure 4. Design of CRISPR/Cas9-mediated σ^{54} deletion in *C. beijerinckii* NCIMB 8052 and phenotypic comparison of wild-type and mutant σ^{54} complementation. **(a)** *sigL* (Cbei_0595) genomic region with CRISPR/Cas9 genome editing design. **(b)** Verification by colony PCR of *sigL* deletion with primers RH129 and RH130 encompassing the deleted region. Expected band sizes are 1406 (wild-type) and 670 ($\Delta sigL$) bp. **(c)** Comparative analysis of fermentation capacities of *C. beijerinckii* NCIMB 8052 wild-type, $\Delta sigL$ and complemented strains. **(d)** Growth comparison of *C. beijerinckii* NCIMB 8052 wild-type, $\Delta sigL$ and complemented strains after 24 hours of fermentation in 2YT, 2YTG and 2YTL liquid media. gRNA: guide RNA. Error bars indicate the standard deviation of triplicate experiments.

A genome editing strategy based on the one described by Wasels *et al.*³² was undertaken. Briefly, an anhydrotetracycline-inducible system allows the selection of double allelic exchange events between the clostridial DNA and a user-defined editing template, by using the Cas9/gRNA complex to target the unmodified bacterial chromosome. Double strand breaks thus only occur in the wild-type or single allelic exchange cases, resulting in cell death.

A plasmid based on the pCas9_{ind} vector was constructed to comprise, in addition to the inducible *cas9* gene, an anhydrotetracycline-inducible gRNA expression cassette and a gene editing template designed to delete most of the *sigL* gene (i.e. gene truncation results in a 13 AA protein, Fig. 4a). Introduction of this plasmid in *C. beijerinckii* NCIMB 8052 and induction of the CRISPR/Cas9 machinery permitted the selection of $\Delta sigL$ clones (Fig. 4b, Supplementary File S7).

One of those $\Delta sigL$ clones was subsequently complemented with the pFW- $\sigma 54$ and pFW- $\sigma 54$ -AA plasmids, the latter allowing the expression of the S336F-mutated σ^{54} .

Fermentation assays revealed that solvent production was drastically altered in the $\Delta sigL$ strain, and that a wild-type phenotype could only be restored with pFW- $\sigma 54$ (Fig. 4c, Supplementary File S7). Complementation with pFW- $\sigma 54$ -AA notably appeared to have no effect on acid reassimilation and solvent production when compared to the $\Delta sigL$ empty vector control strain. Besides, all of those strains displayed a resistance to heat shock (cells scraped from one-week-old plates, 80 °C for 10 min, data not shown), highlighting their retained capacity to correctly form spores. Lastly, $\Delta sigL$ mutant growth appeared to be highly inhibited in lactose-based medium, similarly to the AA strain (Fig. 4d). Cellobiose uptake however did not seem to be impacted in this strain (data not shown).

In addition to confirming our hypothesis on the role of σ^{54} for solventogenesis and sugar utilization, deleting *sigL* in the NCIMB 8052 model strain allowed us to expand our conclusions regarding this transcriptional factor to other microorganisms belonging to the *C. beijerinckii* species.

Discussion

In this study, the allyl alcohol mutant generated from *C. beijerinckii* DSM 6423 by Máté de Gerando *et al.*⁹ was genetically characterized by a forward genetic approach. Complementation assays demonstrated that the AA phenotype is due to a unique point mutation in the CIBE_0767 *sigL* gene encoding the σ^{54} transcriptional factor. This mutation leads to the substitution of a highly conserved serine in the HTH domain by a phenylalanine residue (S336F). Given the central role of the HTH domain in DNA recognition^{37,38}, this mutation is likely to

strongly impede the σ^{54} -RNA polymerase complex binding to promoter sequences and thus to inhibit transcription of the σ^{54} -regulon. If the effect of this particular mutation on σ^{54} functionality has not been described so far, Coppard and Merrick³⁸ performed targeted mutagenesis of the corresponding serine of the HTH domain of *K. pneumoniae*. Its substitution by most residues (especially large ones like lysine or tryptophane) completely shut down σ^{54} -mediated transcription, suggesting this serine is crucial for a functional activity, which supports our hypothesis that σ^{54} is inactivated in the AA strain.

The impact of the S336F mutation in the σ^{54} sequence was experimentally investigated by introducing expression plasmids of either the wild-type or the S336F version of σ^{54} in a $\Delta sigL$ mutant of the model strain *C. beijerinckii* NCIMB 8052. This mutant displayed a phenotype similar to the AA strain, solventogenesis being severely impaired, which resulted in a butyrate-orientated metabolism. Complementation with pFW- σ^{54} fully restored solventogenesis, while the introduction of the pFW- σ^{54} -AA vector in the $\Delta sigL$ strain had no effect. These experiments overall demonstrated that the S336F mutation completely inactivates σ^{54} and are thus consistent with our previous results.

The deleterious effects of allyl alcohol on alcohol-producing microorganisms (yeast^{39–41}, *E. coli*⁴²) and its tendency to generate spontaneous mutations have been reported decades ago. Reverse activity of alcohol dehydrogenases catalyzes the formation of the highly toxic acrolein molecule from allyl alcohol, enabling the selection of alcohol dehydrogenase deficient mutants. In *Clostridia*, similar work was pioneered by Dürre and colleagues¹⁰, who obtained *C. acetobutylicum* mutants unable to produce butanol. Interestingly, butyraldehyde dehydrogenase activity was drastically reduced in these strains. Given the *in silico* σ^{54} -targeted promoter predictions and the expression pattern of the *ald* gene in the WT and AA strains, σ^{54} apparently does not control the butyraldehyde dehydrogenase genes transcription in the DSM 6423 strain. The supposed inactivation of σ^{54} in the AA mutant rather seems to lead to a decreased expression of two major alcohol dehydrogenases genes, resulting in a phenotype close to what was observed in the *C. acetobutylicum* corresponding mutants.

Acetate and butyrate are normally produced during the acidogenic phase and are further consumed during solventogenesis⁴³. In the AA mutant, increased acid production results in a substantial drop of the pH which may prematurely cause cell growth to abort. qPCR data revealed that some normally highly expressed alcohol dehydrogenase genes were underexpressed in the AA strain, which suggests a link between the increase of acid production and the partial loss in butanol production capacity. The significant upregulation of those genes transcription when *sigL* was overexpressed in both the WT and AA strains further consolidates this hypothesis.

Isopropanol production was also abolished in the AA strain but its precursor - acetone - was still detected. As expected, expression of the sole secondary alcohol dehydrogenase (s-ADH, encoded by the CIBE_3470 gene), which catalyzes the acetone-to-isopropanol reaction, was highly affected in this strain. Being linked to acid consumption, acetone formation indicates that butyrate and acetate uptake still occurs. In the DSM 6423 wild-type strain, the resulting acetyl-CoA and butyryl-CoA are further reduced into acetaldehyde/butyraldehyde and then ethanol/butanol. Because alcohol synthesis is drastically reduced in the AA strain, this implies that most of the acetyl-CoA and butyryl-CoA are converted back to acetate and butyrate first by the CtfA/B CoA-transferase^{44,45} and then by phosphotransbutyrylase/acetylase - butyrate/acetate kinase pathway^{46–51}. This assumption would be supported by the increased (acetone + isopropanol):butanol ratio in the AA strain (close to 1:1, versus 1:3 in the wild-type). Indeed, this ratio is expected to be higher if butyryl-CoA is preferentially used to synthesize butyrate instead of butanol.

Among acidic products, butyrate synthesis seems to be preferred over acetate production in the AA strain. Acetate was indeed consumed in most - but not all - fermentation experiments (i.e. final concentration in the fermentation broth less than the 2.4 g/L contained in the Gapes medium) involving the AA mutant or the NCIMB 8052 $\Delta sigL$ strain, which also supports the hypothesis that acid uptake does occur. This also suggests that the butyryl-CoA synthesis operon (i.e. genes linking acetyl-CoA and butyryl-CoA, namely: *hbd*, *crt*, *bcd*, *etfA*, *etfB*^{52–54}), is still constitutively expressed in the AA mutant, similarly to the WT strain⁸. Besides, overexpression of the acid reassimilation pathway (CtfA/B, s-ADH) in the AA strain did not increase acid consumption. In accordance with *in silico* predictions, our data thus strongly suggest that σ^{54} main mode of action consists in driving the regulation of solventogenesis by modulating alcohol dehydrogenases expression and not by interfering on the bacterial acid uptake capacity.

σ^{54} was also shown to control sugar uptake and usage, consistently with what Nie *et al.* predicted for the *Clostridium* genus⁵⁵. In particular, growth was limited on cellobiose and lactose in the AA strain, though cellobiose inhibition could only be observed on minimal medium. This may be explained by the existence of another cellobiose assimilation pathway in the DSM 6423 genome. In the NCIMB 8052 strain, *sigL* deletion was demonstrated to impact lactose uptake, confirming that σ^{54} is involved in sugar uptake and/or metabolism in *C. beijerinckii*.

σ^{54} belongs to a unique category of sigma factors, being evolutionary distinct to the σ^{70} family, which comprises all of the other sigma factors^{56,57}. This difference implies a very different mode of action for the σ^{54} -RNA polymerase complex: unlike its σ^{70} counterpart, the holoenzyme is unable to initiate transcription without the help of an enhancer binding protein (EBP)²². EBPs are often acting several hundred of base pairs upstream of the σ^{54} -holoenzyme deposition sites and contain a central AAA+ domain. The latter provides, through ATP hydrolysis, the energy necessary for structural rearrangements within the σ^{54} -bound holoenzyme, which in turn allows transcription initiation. EBP control on σ^{54} -dependant transcription is mainly exerted by a signal-sensing domain, which upon specific environmental stimuli activates or represses its ATPase activity²². This enhancer-dependent system offers a very tight control on gene expression, and σ^{54} regulons are often associated with various biological processes that require a stringent control (e.g. virulence^{58–60} or biofilm formation^{61–63}). Multiple σ^{54} EBPs were found in *C. beijerinckii* genomes and are thus likely to be the primary effectors of σ^{54} -mediated regulation. Indeed, RNA-seq analysis in the *C. beijerinckii* DSM 6423 wild-type strain⁸ shows that *sigL* is not differentially expressed over the time-course of a batch fermentation, whereas its target solventogenic genes (CIBE_2050, CIBE_2622,

Strains and plasmids	Relevant characteristics	Source or reference
<i>Clostridium beijerinckii</i>		
DSM 6423	Isopropanol-butanol producer	DSMZ
AA	Allyl alcohol-resistant strain obtained by random mutagenesis	Máté de Gerando <i>et al.</i> (2016)
NCIMB 8052	Acetone-butanol producer	NCIMB
NCIMB 8052 $\Delta sigL$	Markerless deletion of <i>sigL</i>	This study
<i>Escherichia coli</i>		
NEB 10- β	Cloning strain	NEB
INV110	<i>dam</i> ⁻ <i>dcm</i> ⁻ strain	Invitrogen
Plasmids		
pFW01	<i>Em</i> ^R , ColE1 origin; pCB102 origin	Wasels <i>et al.</i> (2017)
pEC750C	<i>Cm</i> ^R , ColE1 origin, pIP404 origin	Wasels <i>et al.</i> (2017)
pCas9 _{con}	pFW01 derivative with <i>cas9</i> (constitutive minipTh1 promoter)	Wasels <i>et al.</i> (2017)
pCas9 _{ind}	pFW01 derivative with <i>cas9</i> (inducible TetO2-1 promoter)	Wasels <i>et al.</i> (2017)
pFC06	pEC750C derivative with <i>sadh ctfA ctfB</i> (constitutive <i>thl</i> promoter) insertions	Collas <i>et al.</i> (2014)
pFW- σ^{54}	pFW01 derivative with <i>sigL</i> insertion (endogenous promoter)	This study
pFW- σ^{54} -AA	pFW- σ^{54} bearing the S336F mutation from the AA strain	This study
pCas9- $\Delta sigL$	pCas9 _{ind} derivative with $\Delta sigL$ homology regions and gRNA (inducible TetO2-1 promoter)	This study
pFW-FC06	pFW01 derivative with <i>sadh ctfA ctfB</i> (constitutive <i>thl</i> promoter) insertions	This study

Table 2. Strains and plasmids used in this study. Em, erythromycin; Cm, chloramphenicol.

CIBE_3470) are among the top upregulated genes during the transition from acidogenesis to solventogenesis, which suggests that activation of their transcription is mediated by other factors (i.e. the enhancer binding proteins). RT-qPCR data also suggest that, at least in some cases, expression of *sigL* is regulated, which might provide an additional mean to regulate alcohol synthesis and/or sugar uptake. A well-known example of EBP-related regulation is the σ^{54} -driven transcription of the levanase operon in *B. subtilis*, which encodes an enzymatic and transport machinery allowing the degradation and consumption of fructose polymers^{64,65}. The enhancer binding protein LevR was shown to mediate the activation and repression of the expression of this operon by a complex interplay with the PTS system⁶⁶. In *C. beijerinckii*, the exact stimuli recognized by EBPs and underlying regulatory mechanisms that allow transcription of σ^{54} target genes nonetheless still remain to be clarified.

In bacteria, the housekeeping sigma factor (e.g. σ^{70} in *E. coli*) integrates RNA polymerase and drives transcription of most housekeeping genes. However, bacterial genomes encode multiple alternative sigma factors which direct RNA polymerase to different promoters, providing a simple but broadly exploited strategy to regulate genetic expression^{67,68}. In solventogenic *Clostridia*, alternative sigma factors have mainly been highlighted for their crucial role in the regulation of the multi-stages sporulation cascade⁶⁹⁻⁷¹. In *C. beijerinckii*, no sporulation-specific targets could be found in the predicted σ^{54} regulons, and σ^{54} inactivation did not impede the sporulation cascade, which underlines its role as a carbon-specific regulator in this bacteria.

In summary, we described σ^{54} as a master regulator of solventogenic pathways at the species level in *C. beijerinckii*. This transcriptional factor was also found to control sugar consumption and is therefore an essential controller of carbon metabolism in *C. beijerinckii*.

Material and Methods

Strains, media and culture conditions. Strains and plasmids used in this study are presented in Table 2.

Clostridium beijerinckii was grown in liquid 2YTG (per liter: 16 g tryptone, 10 g yeast extract, 5 g NaCl, 20 g glucose, pH 5.2). Solid media was prepared with 15 g/L agar and less glucose (5 g/L). Strains were cultivated in an anaerobic chamber (Bactron) at 34 °C without shaking.

Escherichia coli was grown in liquid or solid LB media, in aerobic conditions at 37 °C with 180 rpm agitation when necessary.

Whole-genome sequencing. Genomic DNA of the AA strain was purified with the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) and was subsequently sequenced on an Illumina MiSeq sequencer (2 × 250 paired-end reads). Mutations were detected by read mapping against the *C. beijerinckii* DSM 6423 genome⁸ using Geneious R10⁷².

Predictions of σ^{54} -regulons. Genomes of the *C. beijerinckii* DSM 6423, NRRL B-598 and NCIMB 8052 strains were scanned for the σ^{54} consensus motif (TGGCANNNNNTTGCW¹³, no mismatches allowed) using Geneious R10⁷². Genes were considered potentially regulated by σ^{54} if motifs were found up to 500 bp upstream their coding sequences. Predicted candidate genes/operons were then compared in the three strains using the synteny tool from MaGe platform⁷³.

Fermentation assays and analytical methods. For fermentation assays, modified Gapes medium⁷⁴ was used. This medium contains per liter: 2.5 g yeast extract, 1 g KH₂PO₄, 0.6 g K₂HPO₄, 1 g MgSO₄, 7 H₂O, 6.6 mg FeSO₄, 7 H₂O, 0.1 g para-aminobenzoic acid, 2.9 g ammonium acetate, 60 or 80 g glucose. The medium was supplemented with erythromycin (20 µg/mL) when appropriate.

Fermentations were performed in triplicate. For each biological replicate, several clones isolated in 2YTG agar plates were used to inoculate 5 mL Gapes medium. These precultures were carried out at 34 °C overnight in the anaerobic chamber. Serum flasks containing 20 mL Gapes medium were then inoculated with 2 mL of preculture and sealed with rubber stoppers. A pressure relief valve system was punctured through the rubber stoppers to prevent overpressure, and the serum bottles were incubated 48–72 hours at 34 °C with agitation outside of the anaerobic chamber.

After OD₆₀₀ (UV-1800 spectrophotometer, Shimadzu) was measured, samples from the fermentation were centrifuged 5 min at 8000 g. The supernatant was diluted with an internal standard (1-propanol: final concentration 0.5 g/L). Metabolites concentrations in the supernatant were quantified by chromatography.

Gas chromatography (Porabond-Q column from Agilent technologies, 25 m length, 0.32 internal diameter, 5 µm film thickness coupled to a flame ionization detector) was performed to determine solvent concentrations. Helium was used as a carrier gas at a flowrate of 1.6 mL/min. Column was gradually heated from 50 °C to 250 °C in a 30 min run.

Acid concentrations were quantified by HPLC (Aminex HPX-87H from Biorad coupled to a Spectra System RI 150 refractometer and a Waters 2487 Dual λ UV detector set at 210 nm). 0.01 M sulfuric acid mobile phase was used at a flowrate of 0.6 mL/min. Column temperature was set at 60 °C.

Residual sugar quantities were determined by HPLC using an Aminex HXP-87P (Bio-Rad) coupled to a Varian 350 RI refractometer for detection. Water was used as a mobile phase at a flowrate of 0.4 mL/min. When quantifying sugar concentration for 2YT-based samples, a Micro-Guard De-Ashing Refill cartridge (Bio-Rad) was added to the chromatographic system for prior desalting of the samples. Column temperature was set at 80 °C.

Plasmid construction and transformation. All primers used in this study are listed in Supplementary File S6.

The *sigL* gene (CIBE_0767) from *C. beijerinckii* DSM 6423 together with its endogenous promoter were amplified by PCR with primers RH077 and RH078. The pFW01 backbone as well as the thiolase terminator were amplified by PCR from plasmid pCas9_{con}³² with primers RH086 and RH087. Those two amplicons were subsequently assembled by Gibson assembly (NEB)⁷⁵ to yield pFW-σ54. From this plasmid, pFW-σ54-AA was obtained by site-directed mutagenesis with primers RH136 and RH137.

The *sadh* gene (CIBE_3470) from *C. beijerinckii* DSM 6423 and the *ctfA* and *ctfB* genes from *C. acetobutylicum* ATCC 824 were amplified by PCR with primers MKz01 and MKz02 from plasmid pFC006²⁹. The resulting PCR product was cloned between the KpnI and Sall restriction sites to obtain the pFW-FC06 vector.

For the deletion of *sigL* in *C. beijerinckii* NCIMB 8052, two homology regions and an anhydrotetracycline-inducible gRNA expressing cassette were first synthesized by BaseClear in a pUC57 plasmid. Homology regions were designed to delete a 736 bp fragment in the *sigL* gene, yielding a truncated ORF (13 amino acid residues instead of 463). This cassette was amplified with primers RH125 and RH126 and subsequently cloned into the pCas9_{ind} vector³² at the XhoI restriction site. The gRNA protospacer (designed using Genious R10)⁷² was then introduced in the resulting vector by Golden Gate assembly at the BsaI restriction site with primers guide_{sigL}_fwd and guide_{sigL}_rev, yielding pCas9-Δ*sigL*.

The plasmids were then isolated and electroporated into *C. beijerinckii* as previously described^{76,77}. *dam*⁺ *dcm*⁺ DNA and *dam*⁻ *dcm*⁻ DNA were used for *C. beijerinckii* NCIMB 8052 and DSM 6423, respectively.

qPCR assays. Starting from fresh precultures, 20 mL bacterial cultures were carried out anaerobically in duplicates for 16 hours at 34 °C and 120 rpm, starting with an initial OD₆₀₀ value of ≈0.1. Ten mL samples were stabilized on ice by addition of a cold stop solution (ethanol:acid phenol at 1:18 ratio), centrifuged, and the cell pellets were frozen at -80 °C.

TRI reagent (Ambion) was further used to lyse the cells. RNAs were then extracted with the Direct-zol RNA purification kit (Zymo Research). After DNase treatment (Turbo DNase, Invitrogen), RNAs were column-purified with the RNA Clean & Concentrator 25 kit (Zymo Research).

For reverse transcription, 100 ng or RNAs were used in 20 µL reactions (iScript cDNA Synthesis Kit, Bio-Rad). Of the resulting cDNAs, 0.5 µL were then used for qPCR (SsoAdvanced Universal SYBR Green Supermix, Bio-Rad) along with adequate controls (no RT controls, positive controls on gDNA, no template controls). Primers are given with their corresponding qPCR efficiency in Supplementary File S6.

qPCR data were analyzed with the ΔΔC_q method, using *gapC* (CIBE_0769) as a housekeeping gene control and taking into account reaction efficiencies.

CRISPR/Cas9 genome edition. We designed a CRISPR/Cas9 approach to inactivate *sigL* in the NCIMB 8052 strain. An inducible system based on anhydrotetracycline (aTc) addition was used, based on the pCas9_{ind} vector previously used in *C. acetobutylicum* ATCC 824³². Homology regions for genome editing were designed to delete most of the *sigL* gene, resulting in a severely truncated protein (13 amino acid residues, versus 463 for the full gene; Fig. 4a). Following insertion of those homology sequences and of an anhydrotetracycline inducible gRNA cassette, the resulting pCas9-Δ*sigL* plasmid was introduced into *C. beijerinckii* NCIMB 8052 by electroporation. Δ*sigL* mutants were obtained similarly to the method described by Wasels *et al.*³². Briefly, transformants resuspended in liquid 2YTG (pH 5.2, 20 g/L glucose) and serially diluted for spotting onto 2YTG plates

containing erythromycin (20 µg/mL) and anhydrotetracycline (50 ng/mL). Isolated colonies were tested by colony PCR with primers RH129 and RH130, encompassing the homology regions in the genome. Positive mutants were subsequently cured by streaking them twice on 2YTG plates supplemented with 50 ng/mL anhydrotetracycline. Plasmid presence in isolated colonies was next tested on 2YTG plates containing erythromycin (20 µg/mL), and erythromycin-sensitive clones were selected for further analysis.

Sporulation assays. Sporulation assays were based on survival to heat shock, as similarly described⁷¹. Briefly, triplicates precultures were carried out overnight in 2YTG medium. The following day, serum bottles containing 20 mL 2YTG (15 g/L glucose, pH 6.8) were inoculated with 2 mL of preculture and subsequently sealed. After 5 days at 34 °C without agitation, 1 mL of culture was heat shocked (10 min, 80 °C) and serially diluted. 5 µL spots were made for each dilution on 2YTG plates, which were then incubated overnight at 34 °C.

Sugar utilization assays. For liquid sugar utilization tests, liquid 2YTG (pH 6.8) was used, as well as 2YTL (pH 6.8, 20 g/L lactose) and 2YT (pH 6.8, without purified carbon source). Precultures were carried out overnight from fresh colonies in 2YTG medium (pH 6.8) containing 5 g/L glucose and 20 µg/mL erythromycin at several dilutions (up to 10⁻⁴ dilution factor). The following day, fresh precultures were used to inoculate 20 mL (AA strain; serum bottles)/5 mL (Δ sigL strain; 24 deep-well plates) of 2YTG, 2YTL or 2YT media with or without (as needed) erythromycin 20 µg/mL at 0.01 units of OD₆₀₀. Samples were taken after 24 and 96 hours, and sugar concentration was quantified as described above.

Minimal medium (CDM) was used to visualize sugar uptake inhibition by the AA strain. This medium, described by Vasconcelos *et al.*⁷⁸, was supplemented with 15 g/L agar and contained 20 g/L glucose, lactose or cellobiose. Single colonies picked on CDM glucose (5 g/L) plates were serially diluted in water. Spots were made with 5 µL of the 10⁻¹, 10⁻², 10⁻³ and 10⁻⁴ dilutions on minimal medium plates containing glucose, cellobiose or lactose (20 g/L). Plates were visualized after 24 h (glucose, cellobiose) or 48 h (lactose).

Data Availability

Raw reads corresponding to the genome sequencing of the AA mutant have been deposited to the Sequence Read Archive (SRA) database (<https://www.ncbi.nlm.nih.gov/sra>) with the BioProject accession number PRJ-NA530065.

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Acknowledgements

We are grateful to Michelle Kuntz for constructing the pFW-FC06 plasmid and to Gwladys Chartier for technical assistance.

Author Contributions

R.H., N.L.F. and F.W. designed the study. R.H. performed experiments on *C. beijerinckii* DSM 6423 and AA strains. M.B.-L. performed experiments regarding the *C. beijerinckii* NCIMB 8052 strain. R.H., M.B.-L., N.L.F. and F.W. wrote the paper.

Additional Information

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41598-019-43822-2>.

Competing Interests: The authors declare no competing interests.

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