Isolation of a new butanol-producing *Clostridium* strain: High level of hemicellulosic activity and structure of solventogenesis genes of a new *Clostridium saccharobutylicum* isolate ☆

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Abstract

New isolates of solventogenic bacteria exhibited high hemicellulolytic activity. They produced butanol and acetone with high selectivity for butanol (about 80% of butanol from the total solvent yield). Their 16S rDNA sequence was 99% identical to that of *Clostridium saccharobutylicum*. The genes responsible for the last steps of solventogenesis and encoding crotonase, butyryl-CoA dehydrogenase, electron-transport protein subunits A and B, 3-hydroxybutyryl-CoA dehydrogenase, alcohol dehydrogenase, CoA-transferase (subunits A and B), acetoacetate decarboxylase, and aldehyde dehydrogenase were identified in the new *C. saccharobutylicum* strain Ox29 and cloned into *Escherichia coli*. The genes for crotonase, butyryl-CoA dehydrogenase, electron-transport protein subunits A and B, and 3-hydroxybutyryl-CoA dehydrogenase composed the *bcs*-operon. A monocistronic operon containing the alcohol dehydrogenase gene was located downstream of the *bcs*-operon. Genes for aldehyde dehydrogenase, CoA-transferase (subunits A and B), and acetoacetate decarboxylase composed the *sol*-operon. The gene sequences and the gene order within the *sol*- and *bcs*-operons of *C. saccharobutylicum* Ox29 were most similar to those of *Clostridium beijerinckii*. The activity of some of the *bcs*-operon genes, expressed in heterologous *E. coli*, was determined.
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Introduction

A resurgence of interest in the butanol-producing *Clostridia* was recently observed, caused by the growing demand for alternative liquid energy carriers from renewable sources. Butanol has the potential to replace gasoline gradually, as well as diesel or kerosene,

due to its high energy content, miscibility with other fuels, octane improving power, low volatility and other attributes beneficial to combustion engines [24].

During the last century, the technological process of clostridial solvent fermentation was the second largest industrial fermentation process besides yeast-based production of ethanol until it failed to compete with the booming oil industry in the 1980s [1]. This process is usually referred to as ABE fermentation, after its main components of acetone, butanol and ethanol. Four distinct species of clostridia were identified among the industrial production strains: *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharoperbutylacetonicum*, and *Clostridium saccharobutylicum* [15]. *C. saccharobutylicum* (former *C. acetobutylicum* NCP262) is an obligate anaerobic spore-forming solventogenic bacterium. It was differentiated from *C. acetobutylicum* species on the basis of 16S rDNA sequencing and DNA–DNA reassociation [16]. Industrial strains derived from *C. saccharobutylicum* NCP262 were amongst the most successful saccharolytic, solvent-producing clostridia utilized for the commercial production of solvents. They were used in the ABE plants of South Africa for production of acetone and butanol from molasses [13,14,25]. *C. saccharobutylicum* has, nonetheless, been studied less intensively than *C. acetobutylicum* and *C. beijerinckii*. Little is currently known about the genomic structure and organization of this organism [16]. Only several genes involved in the final steps of the acetone and butanol production of *C. saccharobutylicum* were cloned and identified: the *adh-1*, *hbd*, and *fixB* genes encoding NADPH-dependent alcohol dehydrogenase, 3-hydroxybutyryl-CoA dehydrogenase and a subunit of the electron-transport protein [29,30].

A physical and genetic map of the *C. saccharobutylicum* NCP262 chromosome was constructed. Some of the genes involved in the acid and solvent forming pathway were shown to be clustered in a small region, representing only 2–5% of the NCP262 genome. This region includes the butyryl-CoA synthesis (*bcs*) operon genes, *crt*, *bcd*, *etfB*, *etfA (fixB)*, and *hbd*, encoding enzymes which reduce acetoacetyl-CoA to butyryl-CoA in three consecutive reactions, and the butyrate operon genes, *ptb* and *butK*, involved in the conversion of butyryl-CoA to butyrate [16]. However, these genes (with the exception of *fixB* and *hbd*) had not yet been cloned and sequenced.

In this study, the isolation of new solvent-producing strains of the anaerobic bacterium *C. saccharobutylicum* is described. These bacteria exhibit high hemicellulolytic activity for enhanced conversion of plant biomass. *C. saccharobutylicum* Ox29 genes involved in solventogenesis were identified, isolated, and heterologously expressed in *Escherichia coli*.

Materials and methods

Strains, media and growth conditions

The bacterial strains used in this study were *C. acetobutylicum* DSM792^T (ATCC824) and *C. beijerinckii* DSM791 from the DSMZ (Germany), *C. saccharobutylicum* Ox29, Ox31 and Ox44 from this study, and *E. coli* TOP10F' (Invitrogen, USA) and *E. coli* XL-1 Blue (Stratagene, USA). Reinforced Clostridial Medium (RCM, Merck), grass medium (GRM: 1–6% (w/v) of dried grass in tap water) and minimal salt medium (MSS) [4] were used to maintain and cultivate the strains. Various carbohydrates at a concentration of $60\,g\,L^{-1}$ replaced glucose in MSS medium where indicated. Liquid *Clostridium* cultures were grown anaerobically from spores freshly activated by heating (10 min at 80 °C). Anaerobic conditions for agar plates were obtained in an anaerobic chamber (Coy, USA). *E. coli* cultures were grown in LB medium. Ampicillin ($100\,\mu g\,mL^{-1}$) was added when necessary [23].

Isolation of solvent-producing bacterial strains capable of degrading plant biomass

Samples of soil, plants, silt or decomposed waste were collected from agricultural areas on and around a university campus (Freising, Germany). Anaerobic spore-forming bacteria capable of using plant biomass were isolated according to the method described by Montoya et al. [20] with the following modifications: collected samples after heat inactivation of vegetative cells were enriched for hemicellulolytic and cellulolytic strains by anaerobic growth on media containing plant biomass as the sole carbon source. The enriched cultures were tested for solvent production by gas chromatography. Butanol positive cultures were examined under a microscope for uniformity of shape and size of cells, as well as the presence of spores.

DNA techniques

General DNA manipulation was carried out according to standard protocols [23]. Primers used for DNA-fingerprinting, strain identification, and gene cloning are listed in Table 1. The "puReTaq^{TM}Ready-To-Go^{TM}PCR Beads" (Amersham, USA), Expand Long Range Pack (Roche) kits and KOD-polymerase (Novagene) were used for amplification of bacterial genes. Asymmetrical PCR was performed in accordance with Zverlov et al. [32]. SR-PCR analysis was performed in accordance with Barry et al. [2] and Song et al. [26]. The TOPO TA Cloning kit containing the pCR2.1 vector and the TOPO-XL kit containing the pCR-XL

Table 1. Primers used in the study.

Primers	Sequence ($5' \rightarrow 3'$)	Source	Target strain
16S 23S	GCTGGATCACCTCCTTTC GTTYCCYCATTCGG	SR-PCR [2]	Newly isolated strains (this study)
616V 630R	AGAGTTTGATYMTGGCTC CAKAAAGGAGGTGATCC	16S rDNA [4]	Newly isolated strains (this study)
Ald for Adc rev	TTATATTTACTTTTTTGGTTTAATGGG TTGCTTTTATTTTACTGAAAGATAATC	*C. beijerinckii* NCIMB8052 *sol* operon: *ald*, *ctfA*, *ctfB*, and *adcA* genes (GenBank CP000721)	*C. beijerinckii* DSM791
BdhAB for BdhAB rev	TTCCTGCCTCACAAGAAATTATG TTCGCGTTATTTTTAGATGCTTC	*C. acetobutylicum* ATCC824: *bdhA* and *bdhB* genes (GenBank AE001437)	*C. acetobutyli-cum* ATCC824
Op for Op rev	ATGAATATAGCAGATTACAAAGGCG ACTTTATTAAAAATCAACTTCTGTACC	*C. saccharobutylicum* NCP262: *adh-1*, *hbd*, and *fixB* genes [28]	*C. saccharobuty-licum* Ox29
Ald sol for Ald sol rev	AGATAGGAATGGAATGAACCG AGTCTGATGCATTAAGTTTGG	*C. saccharobutylicum* Ox29: *ald*A gene	*C. saccharobuty-licum* Ox29
pr-bcs-Csa1 [1] pr-bcs-Csa2 [2] bcs-Csa4r [3] sacch-adh for + [4]	TTATGAATGGGATTTAAGACAAG AATAGGCAAGTTTAAGGGAGG TTTATTAGAAATCAACTTCTGTACC AATCATATGATTGCTTAAGGTAC	*C. saccharobutylicum* Ox29 DNA fragments containing *bcs*-operon and *adhA* gene with *bcs*- and *adhA*-promoters [1,3]; without *bcs*-promoter [2,3]; *adhA* gene with *adhA* promoter [3,4]	*C. saccharobuty-licum* Ox29

vector (Invitrogen, USA) were used for cloning and expression steps with *E. coli* TOP10F' competent cells.

BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and DNASIS/PROSIS for Windows (Hitachi Software Engineering) software packages were used for sequence data analysis.

Analytical methods

The production of solvents by *Clostridium* strains was determined quantitatively by gas chromatography. Cells were grown anaerobically without shaking at 37 °C on MSS medium until the completion of fermentation. For measurements, a GC-2010 apparatus (SHIMADZU, Japan) equipped with a Phase-Stabilwax-DA column (60 m × 0.32 mm × 0.5 mm) and a flame ionisation detector was used with nitrogen as the carrier gas. Analysis of the chromatograms was carried out using GC-Solution software (SHIMADZU). The measurements were performed at least three times, with an observed measurement inaccuracy of less than 3%.

Enzyme assays

Activities of the enzymes responsible for solventogenesis were determined in cell extracts in accordance with Boynton et al. [5], Lehman and Thorpe [18], Palosaari and Rogers [22], and Youngleson et al. [28,30].

Activities of the extracellular glycosyl hydrolases were measured in cell-free culture liquid of cells 12 h after inoculation in fresh medium, as described earlier in Berezina et al. [4]. Reducing sugars liberated from polysaccharides were determined by the dinitrosalicylic acid method [10]. Protein concentrations were determined by the Bradford dye-binding assay [6]. BSA (2 mg mL^{-1} albumin, Pierce) was used as a standard. Substrates for enzyme activity measurements were obtained from Sigma-Aldrich. The measurements were performed at least three times, with an observed inaccuracy of less than 10%.

Results

Isolation of solvent-producing bacterial strains growing on plant biomass

A total of 52 samples was collected from the environment and screened for solvent-producing bacteria capable of utilizing plant biomass for solvent production. Acetone, butanol or butyric acid was detected in cultures from potato, silt and rye grain samples. After single-colony purification on plates, 48 colonies were isolated. The cells of all isolates were straight rods capable of forming endospores.

The new isolates were subjected to DNA-fingerprinting (SR-PCR analysis based on 16S-23S rDNA spacer region polymorphism) [2]. Four DNA patterns were identified (data not shown). At least two representative strains were randomly selected from each of the four patterns for 16S rDNA amplification and sequencing.

The obtained sequences were analyzed by a comparison with the GenBank database. Characteristics of the 48 clostridial strains belonging to four SR-PCR patterns are shown in Table 2. Pattern A (13 isolates from potato) was related to *C. saccharobutylicum* (99% identity in 16S rDNA sequence), and the strains from this group produced acetone and butanol. Patterns B (four isolates from potato) and C (26 isolates from silt) were sister groups of the butyrate-producing species *C. butyricum*. The fourth, pattern D, contained five butyrate-producing clostridial isolates from rye grain that showed low similarity to an unidentified *Clostridium* sp. (AY082483) with a 16S rDNA gene identity of 94% to *C. acetobutylicum* ATCC824.

Phenotype, solvent production, and polysaccharolytic activity of the newly isolated *C. saccharobutylicum* strains

Growing liquid cultures contained straight, motile, long and short rods, single or joined in pairs and short chains. Cells in older cultures transformed into cigar-shaped clostridia, probably due to granulose accumulation. The shape of the cells corresponded to the growth phase of the culture: exponential growth and acid accumulation (rods), metabolic shift or solvent production (clostridia). At the end of fermentation the clostridial cells formed endospores. Colonies on RCM agar were slightly irregular, had a diameter of 1.5–3 mm, were creamy in color and had a smooth surface. Optimal growth was observed at temperatures between 25 and 37 °C and a pH between 6.0 and 7.0.

The newly isolated *C. saccharobutylicum* strains produced butanol, acetone, ethanol, acetic and butyric acids, H_2, and CO_2. Riboflavin was not produced. The maximum level of butanol production at 37 °C on 6% (v/w) glucose medium was observed in cultures of *C. saccharobutylicum* Ox29, *C. saccharobutylicum* Ox31, and *C. saccharobutylicum* Ox44 with 9.2, 8.6, and 9.7 g L^{-1}, respectively. Acetone production for these strains was about 0.4–2 g L^{-1}, and ethanol production 0.1–0.2 g L^{-1}. i-Propanol was not produced in detectable amounts under these conditions.

The newly isolated *C. saccharobutylicum* Ox29, Ox31, and Ox44 strains fermented mono-, di-, and poly-saccharides, particularly xylose, glucose, cellobiose, maltose, starch, xylan, and plant biomass (grass). The polysaccharolytic activity of these strains was investigated after growth on different substrates. None of the strains produced true cellulase activity by hydrolysing crystalline cellulose (Avicel). The maximum level of

Table 2. Characteristics of the 48 clostridial strains belonging to four SR-PCR groups.

SR-PSR patterns	Number of new isolates	Most related organism	16S rDNA gene, Acc. no.	16S rDNA gene identity, %[a]	Butanol production	Source of sample
A	13	*C. saccharobutylicum* NCP262	U16147	99	+	Potato field
B	4	*C. butyricum* RCEB	EU621841	99	–	Potato field
C	26	*C. butyricum* W4	DQ831126	99	–	Silt
D	5	*Clostridium* sp. 44a-T5zd	AY082483	98	–	Rye grain

[a]The percentage shows the similarity to the closest GenBank organism determined using nearly full-length sequences of bacterial 16S RNA genes from two selected isolates of each group.

Table 3. Extracellular hemicellulase activity in *C. saccharobutylicum* Ox29 culture supernatants grown on different carbohydrate substrates.

Activity on	Carbohydrate substrate used for growth											
	Glucose		Maltose		Xylose		Starch		Xylan I		Cellobiose	
	U mg^{-1}/U mL^{-1}											
Barley β-glucan	7363	51.5	4485	80.8	4264	85.3	4967	79.5	2834	85.0	3096	154.8
Xylan I	0	0	81.0	1.5	88.9	1.8	95.2	1.5	211	6.3	639	32.0
Xylan II	8.7	0.06	127.3	2.3	123.6	2.5	223.2	3.6	257	7.7	781	39.1
CMC	0	0	61.4	1.1	61.4	1,2	59.4	1.0	149	4.5	106	5.3
PASC	0	0	0.9	0.02	1.6	0.03	0	0	47	1.4	55	2.8

Values are given in U mg^{-1} protein and U mL^{-1} culture supernatant. Xylan I and xylan II are oat spelt and larchwood xylan, respectively; PASC is phosphoric acid-swollen cellulose.

hemicellulase activity was detected for *C. saccharobutylicum* Ox29, and this strain was selected for further investigation. After growing on glucose, maltose, xylose, cellobiose, xylan and starch the strain produced extracellular enzymes hydrolysing β-glucan, xylan I, xylan II, CM-cellulose (CMC) or phosphoric acid-swollen cellulose (PASC) (Table 3). The activity of glucose-grown *C. saccharobutylicum* Ox29 on xylan, CMC and PASC was quite low or even absent, in contrast to the same activities of the strain grown on other sugars or polysaccharide substrates. However, activity on barley β-glucan was obviously constitutively expressed, whereas the other enzyme activities were only detected if glucose was not the carbohydrate source.

C. saccharobutylicum Ox29 was deposited in the Russian National Collection of Industrial Microorganisms at the Institute of Genetics and Selection of Industrial Microorganisms (VKPM, B-10183).

Identification and cloning of the genes responsible for solventogenesis

Genomic DNA of the type strains of *C. acetobutylicum* ATCC824 and *C. beijerinckii* DSM792 was used as a template for PCR amplification. The *C. beijerinckii* sol-operon containing the *ald*, *ctfA*, *ctfB*, and *adc* genes was amplified with specific primers (Table 1) and *C. beijerinckii* chromosomal DNA as template. The PCR fragment obtained was verified by sequencing and labelled using digoxygenin (DIG).

Chromosomal DNA of *C. saccharobutylicum* Ox29 was digested with BamHI, EcoRI, PstI or HindIII restriction endonucleases and subjected to Southern blot hybridization using the labelled PCR fragment from the *C. beijerinckii* sol-operon genes. Two HindIII DNA fragments (1.8 and 3.5 kb) hybridized with the labelled 3.5 kb *C. beijerinckii* DNA fragment (data not shown). A genomic library of *C. saccharobutylicum* Ox29 was constructed in vector pUC19 using HindIII digested chromosomal DNA. Recombinant clones were re-hybridized with the same probe. Plasmid DNA was isolated from positive clones, and the inserts were sequenced. As a result, four open reading frames with homology to *C. beijerinckii* aldehyde dehydrogenase, CoA-transferase (subunits A and B), and acetoacetate decarboxylase genes were identified in the *C. saccharobutylicum* Ox29 genome and designated *aldA*, *ctfA*, *ctfB*, and *adcA*, respectively. Putative ribosome-binding sites were located upstream of each of these genes. High similarity to the consensus motif of a σ^A-dependent control region (a TTGACA hexamer and a TTTAAT hexamer separated by an 18 bp spacer) was found 139 bp upstream of the *aldA* start codon. A stem-loop structure (AAUAGCCAUUCUGAAU) with a calculated free energy of -19.2 kcal mol^{-1} was identified 33 bp down-stream of the *adcA* stop codon. Another dyad symmetry structure (AACUUAAUGCA) with a calculated free energy of -11.2 kcal mol^{-1} was mapped 11 bp down-stream of the *aldA* stop codon. However, no promoter-like sequences were found upstream of the *ctfA* gene. This suggested that the *aldA*, *ctfA*, *ctfB*, and *adcA* genes of *C. saccharobutylicum* Ox29 comprised an operon (named the sol-operon) homologous to those of *C. beijerinckii*, *C. acetobutylicum*, and *C. saccharoperbutylacetonicum* (Fig. 1A).

C. acetobutylicum ATCC824 *bdhA* and *bdhB* genes encoding two NADH-dependent butanol dehydrogenases were adjacent on the chromosome. *bdhA* and *bdhB* genes were amplified by specific primers (Table 1) with *C. acetobutylicum* genomic DNA as a template. Under the conditions used (standard hybridization buffer, 60 °C) these two genes did not hybridize with *C. saccharobutylicum* Ox29 chromosomal DNA.

Specific primers were designed on the basis of the *C. saccharobutylicum* NCP262 *fixB-hbdA-adh-1* sequence published by Youngleson et al. [28] and used for amplification of the corresponding DNA fragment of *C. saccharobutylicum* Ox29. An amplified fragment was cloned in the TOPO vector and sequenced. The nucleotide sequence contained three open reading frames almost identical to the genes encoding a subunit of the electron-transport protein (*etfA*), the 3-hydroxybutyryl-CoA dehydrogenase (*hbdA*), and the NADPH-linked butanol dehydrogenase (*adhA*) from *C. saccharobutylicum* NCP262. DNA regions located upstream and downstream of the *etfA-hbdA-adhA* fragment were sequenced using an asymmetrical PCR approach [32]. Open reading frames with homology to the genes encoding subunit B of the electron-transport protein (*etfB*), butyryl-CoA dehydrogenase (*bcd*), and crotonase (*crt*) from *C. beijerinckii* NCIMB8052 and *C. acetobutylicum* ATCC824 were found and designated *etfB*, *bcdA* and *crtA*. Therefore, *crtA*, *bcdA*, *etfB*, *etfA*, *hbdA*, and *adhA* genes were clustered on the genome of *C. saccharobutylicum* Ox29 (Fig. 1B), with putative ribosome-binding sites located upstream of each of them. A putative promoter region (a TTGCAA and a TATAAT hexamer separated by a 17 bp spacer) was recognized 130 bp upstream of the *crtA* start codon. A stem-loop structure (AGGACUCUCAAGGUU) with a free energy of -14.2 kcal mol^{-1} was identified 228 bp downstream of the *hbdA* stop codon. An additional dyad symmetry structure (AUCCUUAGUUAAAGUA) with a calculated free energy of -13.8 kcal mol^{-1} was mapped 112 bp downstream of the *etfA* stop codon, although no promoter-like sequences were found upstream of the *hbdA* gene.

It is proposed that the *crtA*, *bcdA*, *etfB*, *etfA*, and *hbdA* genes comprise an operon which is designated as the bcs-operon. The *adhA* gene seems to form a monocistronic operon. The sequences upstream of the
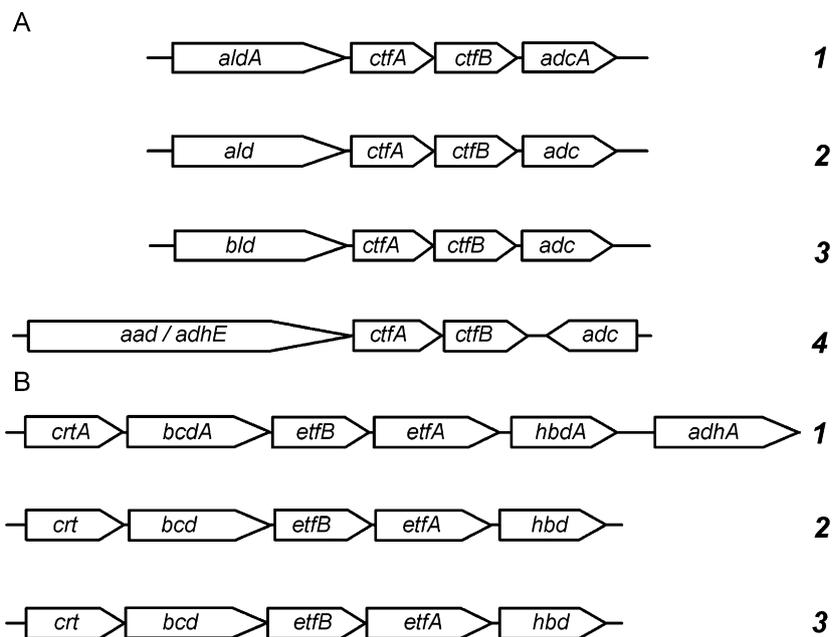
Fig. 1. Organization of solventogenesis genes in four *Clostridia* species. (A) Clostridial *sol*- and *adc*-operons: 1. *C. saccharobutylicum* Ox29; 2. *C. beijerinckii* NCIMB8052; 3. *C. saccharoperbutylacetonicum* N1-4; 4. *C. acetobutylicum* ATCC824. (B) Clostridial *bcs*- and *adhA*-operons: 1. C. *saccharobutylicum* Ox29; 2. *C. beijerinckii* NCIMB8052; 3. *C. acetobutylicum* ATCC824.

C. saccharobutylicum Ox29 *adhA* gene and the *C. saccharobutylicum* NCP262 *adh*-1 gene were aligned and putative *adhA* promoters and RBS were determined by comparative analysis. A putative terminator (AG-GUUCAGUA, calculated free energy $-11.7\,\text{kcal}\,\text{mol}^{-1}$) was identified 34 bp downstream of the *adhA* stop codon. The homology between the enzymes encoded by the *sol*- and *bcs*-operon genes, the acetate decarboxylase, and the alcohol dehydrogenase from four species of *Clostridium* is shown in Table 4. Therefore, the genes potentially responsible for the last steps of solvent formation in *C. saccharobutylicum* Ox29 were identified. Its operon structure was identical to that found in *C. saccharobutylicum* NCP262 and the sequence of the two operons equaled that of the preliminary genomic sequence of the type strain by more than 97% (A. Ehrenreich, personal communication).

Expression of *C. saccharobutylicum* Ox29 solventogenesis genes in *E. coli*

The *aldA* gene from *C. saccharobutylicum* Ox29 was expressed in *E. coli*. Gene *aldA* was additionally amplified by PCR from chromosomal DNA of *C. saccharobutylicum* Ox29 using the primers listed in Table 1, which were designed to include the original *sol*-promoter in the fragment. A TOP-*aldA* plasmid was constructed by cloning the PCR fragment into the pCR2.1 vector. The activity of the recombinant product of the *aldA* gene in *E. coli*/TOP-*aldA* cells in comparison with *E. coli* cells without plasmid was measured in the

physiological direction using acetyl-CoA or butyryl-CoA as the substrate (Table 5).

The cloning of the *C. saccharobutylicum* Ox29 DNA fragment containing the *bcs*-operon and *adhA* gene, controlled by their own promoters, was unsuccessful. This was probably due to the toxicity of the metabolic products for *E. coli* cells. The same genome fragment lacking the *bcs*-promoter was cloned in the pCR-XL vector under the control of the IPTG inducible *lacZ* promoter. Positive clones were obtained and their inserts were checked by sequencing. Crotonase, butyr-yl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydrogenase, and alcohol dehydrogenase activities in the recombinant *E. coli* cells were determined (Table 5). However, alcohol dehydrogenase and butyryl-CoA dehydrogenase activities were not detected.

The DNA fragment containing the *adhA* gene and its promoter region was re-cloned into the pCR-XL vector. However, *E. coli* cells transformed with the resulting TOP-*adhA* plasmid did not express measurable butanol dehydrogenase activity.

Discussion

There are two possible approaches for obtaining an industrial bacterium capable of fermenting renewable lignocellulosic biomass for the production of liquid energy carriers: (1) isolating a bacterium which degrades one or both of the basic polysaccharides in the plant primary cell wall, cellulose and hemicellulose, and

Table 4. The percentage identity/similarity between solventogenesis enzymes from *C. saccharobutylicum* Ox29 and related *Clostridium* strains.

C. saccharobutylicum Ox29	*C. saccharobutylicum* NCP262	*C. acetobutylicum* ATCC824	*C. beijerinckii* NCIMB8052	*C. saccharoper-butylacetonicum* N1-4
Crotonase CrtA (CAQ53134) enoyl-CoA hydratase	nd	Crotonase (NP 349318) **69/85**	Crotonase (YP_001307465) **90/95**	nd
Butyryl-CoA dehydrogenase, BcdA (CAQ53135)	nd	Butyryl-CoA dehydrogenase (NP 349317) **79/89**	Acyl-CoA dehydrogenase domain-containing protein (YP 001307466) **92/96**	nd
Electron transfer flavoprotein beta subunit EtfB (CAQ53136)	nd	Electron transfer flavoprotein subunit beta (NP 349316) **73/85**	Electron transfer flavoprotein, beta subunit-like (YP 001307467) **88/94**	nd
Electron transfer flavoprotein alpha subunit EtfA (CAQ53137)	FIXB (M91817) **98/99**	Electron transfer flavoprotein subunit alpha (NP 349315) **71/82**	Electron transfer flavoprotein, alpha subunit-like (YP 001307468) **88/94**	nd
3-hydroxybutyryl-CoA dehydrogenase HbdA (CAQ53138)	NAD-dependent 3-hydroxybutyryl-CoA dehydrogenase (M31799) **98/99**	3-hydroxybutyryl-CoA dehydrogenase (NP 349314) **79/89**	3-hydroxybutyryl-CoA dehydrogenase (YP 001307469) **93/96**	nd
NADPH-dependent alcohol (butanol) dehydrogenase AdhA (CAQ53139)	NADPH-dependent butanol dehydrogenase (M26941) **96/98**	Bifunctional acetaldehyde-CoA/ alcohol dehydrogenase ADHE1[a] (NP 149199) **40/62**	Iron-containing alcohol dehydrogenase (YP 001309304) 69/82	Butanol dehydrogenase (BAF45463) **27/46**
Aldehyde dehydrogenase AldA (CAQ57983)	nd	Bifunctional acetaldehyde-CoA/ alcohol dehydrogenase ADHE1[b] (NP 149199) **30/52**	Aldehyde dehydrogenase (YP 001310903) **86/92**	Butyraldehyde dehydrogenase (AAP42563) **88/93**
CoA transferase, subunit A, CtfA (CAQ57984)	nd	Butyrate-acetoacetate CoA-transferase subunit A (NP 149326) **71/87**	3-oxoacid CoA-transferase, subunit A (YP 001310904) **88/94**	CoA transferase subunit A (AAP42564) **88/94**
CoA transferase, subunit B, CtfB (CAQ57985)	nd	Butyrate-acetoacetate CoA-transferase subunit B (NP 149327) **70/85**	3-oxoacid CoA-transferase, subunit B (YP 001310905) **81/90**	CoA transferase subunit A (AAP42565) **82/90**
Acetoacetate decarboxylase, AdcA (CAQ57986)	nd	Acetoacetate decarboxylase (NP 149328) **74/83**	Acetoacetate decarboxylase (YP 001310906) **90/93**	Acetoacetate decarboxylase (AAP42566) **90/94**

nd – sequence not determined.

[a] Homology with the *alcohol dehydrogenase* part of the bifunctional alcohol–aldehyde dehydrogenase AdhE1.

[b] Homology with the *aldehyde dehydrogenase* part of the bifunctional alcohol–aldehyde dehydrogenase AdhE1.

ferments the resulting sugars to solvents; and (2) introducing either the polysaccharide degradation genes from a perfect degrader into a solvent producer, or the solvent production pathway genes into a cellulolytic/ hemicellulolytic bacterium. The former method was practised several times following the change of base substrate from starch and sugar to modern, ''second generation'' biotechnology.

Here, we introduce a practicable way to isolate solventogenic bacteria with high cellulolytic or hemi-cellulolytic activity. The endogenous extracellular hydrolytic activity could at least be a partial substitute for (and therefore considerably reduce the amount of) the enzymes to be added for substrate degradation. The new bacteria were isolated from samples taken from various sources of naturally decomposed plant material by direct selection on a medium containing grass as the carbon source. Pasteurization and anaerobic conditions were used to select for clostridia. After single-colony purification, the bacteria producing n-butanol and

Table 5. Specific activity (U mg^{-1}) of the recombinant enzymes, encoded by *crtA*, *hbdA*, and *aldA* genes from *C. saccharobutylicum* Ox29 expressed in *E. coli*.

Activity	Gene	U mg^{-1}
Acetaldehyde dehydrogenase	*aldA*	12×10^{-3}
Butyraldehyde dehydrogenase	*aldA*	10×10^{-3}
Crotonase	*crtA*	72
3-Hydroxybutyryl-CoA dehydrogenase	*hbdA*	3.8
Alcohol dehydrogenase	*adhA*	nd
Butyryl-CoA dehydrogenase	*bcdA*	nd

nd, not detected.

having the highest hydrolytic activity on cellulose or hemicellulose were selected, purified and characterized.

The bacteria were novel *C. saccharobutylicum* strains according to their 16S rDNA sequence (Table 2) and the classification of solventogenic clostridia [27]. A high level of 16S rDNA sequence homology ($>97\%$) has been reported for three related species of solvent-producing clostridia, *C. beijerinckii*, *C. saccharoperbutylacetonicum*, and *C. saccharobutylicum* [14,15]. Deduced amino acid sequences of the *etfA*, *hdbA* and *adhA* genes showed 96–98% identity with the type strain *C. saccharobutylicum* and thus provided additional confirmation for the species affiliation (Table 4).

Hemicelluloses, mainly β-glucans and xylans, comprise up to 40% of plant biomass and are thus a desirable substrate for an industrial process. β-glucanase and xylanase activity of the newly isolated *C. saccharobutylicum* strains were extremely high (see Table 3) and exceeded that of other solvent-producing clostridia strains considerably. For example, the β-glucanase activity of *C. saccharobutylicum* Ox29 grown on glucose was 7363 U mg^{-1} of total protein in the culture liquid (Table 3). As a comparison, the β-glucanase activity of *C. acetobutylicum* ATCC824 grown on various carbohydrate substrates ranged from 0.5 to 7.1 U mg^{-1}, the activity of the former Russian industrial strain *C. acetobutylicum* 7 ranged from 18.9 to 242.7 U mg^{-1}, depending on the growth substrate [4]. Xylanase and CMC activity of *C. saccharobutylicum* Ox29 grown on cellobiose reached 781 and 106 U mg^{-1}, respectively. Even the extracellular enzymatic activities of starch grown cultures (223 and 59 U mg^{-1}) exceeded by far those of the natural Colombian isolate IBUN22A (closest ref. *C. saccharobutylicum* NCP262) that showed 5.4 U mg^{-1} xylanase and 2.6 U mg^{-1} CMC activity when grown on starch [20]. Hence, hemicellulose (including mixed-linkage β-glucan) should be an excellent substrate for the new strain.

The industrial strains belonging to *C. saccharobutylicum* NCP262 typically utilized molasses containing 6–7.5% fermentable sugars, and produced 17–20 g L^{-1}

Table 6. Solvent production of the newly isolated *C. saccharobutylicum* strains in comparison with Russian industrial *C. acetobutylicum* strains and natural Colombian clostridial isolates.

Solvents, g L^{-1} Strain	Butanol	Acetone	Ethanol
C. saccharobutylicum Ox29[a]	9.2	0.4	0.1
C. saccharobutylicum Ox44[a]	9.7	0.9	0.2
C. acetobutylicum 6 [4][b]	7.2	5.1	1.8
C. acetobutylicum 7 [4][b]	8.3	5.8	0.8
C. acetobutylicum VKPM B-4786 [4][b]	6.9	4.9	0.8
IBUN 18A [20][a]	nd	19.1	10.1
IBUN 22A [20][a]	3.0	17.21	0.1
IBUN 62F [20][a]	6.8	8.5	0.9

nd – not detected.
[a]Solvent production on MSS medium with 6% (v/w) glucose.
[b]Solvent production on MSS medium with 6% (v/w) starch.

of solvents with solvent yields of 27–33%, whereas the butanol content ranged from 55% to 74% of the solvents with a B:A:E ratio of 7:2:$<$1 [16,19]. In a first attempt, using batch culture without optimization of media and conditions, the newly isolated *C. saccharobutylicum* strains produced 9–12 g L^{-1} of total solvents in 6% glucose medium with the rate of butanol production averaging 75–80% of the total solvent yield. One could expect that the new strains should produce a high level of solvents during fermentation on hemicellulose-containing media. However, the solvent yield after growth on grass medium was quite low (less than 0.3 g L^{-1}) despite good growth characteristics, such as gas formation or increase in culture turbidity. This can be explained by the presence of inhibiting substances in autoclaved grass or the lack of essential macroelements and microelements in the not yet optimized grass medium.

The solvent ratio produced with the newly isolated *C. saccharobutylicum* strains on glucose was compared with that of the Russian industrial *C. acetobutylicum* strains grown on starch [4,33], and the natural Colombian isolates [20] (Table 6). It should be mentioned that there is a considerable variation in the solvent production and ratio of solvents for different isolates of *C. saccharobutylicum*. This indicates a broad metabolic variability within the species, which should be studied carefully since it could be exploited for metabolic engineering. Of special value is the finding that the new isolates have a high selectivity for butanol and are therefore especially suitable for industrial butanol production.

The genes for the enzymes participating in solventogenesis of the clostridia are coordinately expressed through their organization in operons. Essential components of acetone, butanol, and ethanol synthesis are

encoded by the *sol*-operons of solventogenic clostridia. Here, a complete *sol*-operon was identified for the first time in a strain of *C. saccharobutylicum* and the analysis of the *sol*-operons in all four solventogenic species is now complete. This *sol*-operon comprises the *aldA*, *ctfA*, *ctfB*, and *adcA* genes. The composition and gene order of the *C. saccharobutylicum* Ox29 *sol*-operon is analogous to that of *C. beijerinckii* and *C. saccharoperbutylacetonicum* [17] (Fig. 1A). This is in contrast to *C. acetobutylicum*, in which the *sol*-operon includes the bifunctional aldehyde–alcohol dehydrogenase (*aad*/*adhE*) and CoA-transferase (*ctfA*, *ctfB*) genes [12,21]. The *adc* gene encoding the *C. acetobutylicum* acetoacetate decarboxylase is organized in a monocistronic operon [8,9], and the transcription direction of the *sol*- and the *adc*-operon is convergent. *C. acetobutylicum* is the only solventogenic *Clostridium* known so far to carry an *aad*/*adhE* gene encoding a bifunctional aldehyde–alcohol dehydrogenase in the *sol*-operon. This gene is not present in *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum*, which contain the *ald* gene encoding aldehyde dehydrogenase in the *sol*-operon.

The genes participating in butyryl-CoA synthesis are clustered in the *bcs*-operon, which has an identical gene order in all species of solventogenic clostridia (Fig. 1B) [3,5]. Unlike other species of the solventogenic *Clostridia*, in *C. saccharobutylicum* the alcohol dehydrogenase gene is located adjacent to the *bcs*-operon. The transcription of the genes of the *bcs*-operon and *adh-1* has been investigated in the closest related species *C. saccharobutylicum* (former *C. acetobutylicum*) NCP262 [29,31]. It was demonstrated that the *adh-1* gene located downstream of the *bcs*-operon forms a monocistronic operon with an onset of transcription immediately prior to the solventogenic phase. The *hbd* and *fixB* genes were transcribed throughout the acidogenic and solventogenic phases [31]. Youngleson et al. [29] suggested that the *C. saccharobutylicum* NCP262 *adh-1* gene is regulated in a manner similar to other genes involved in solvent production, which are induced or derepressed prior to solventogenesis, and concluded that the *adh-1* gene may not be the gene responsible for the NADPH-dependent alcohol dehydrogenase activity during the acidogenic and solventogenic phases. Therefore, other genes encoding alcohol dehydrogenases may be present in *C. saccharobutylicum* strains.

Two regions of transcription initiation with start sites 145 (Pl) and 40 bp (P2) upstream of the *adh-l* ATG start codon were detected by Youngleson et al. [29]. These sequences show a strong similarity to the extended consensus sequence for Gram-positive promoters. Comparison of the *hbd-adhA(adh-1)* intergenic region in *C. saccharobutylicum* Ox29 and *C. saccharobutylicum* NCP262 showed 92% sequence identity. An alignment of the Pl (−10) sequences of *C. saccharobutylicum* Ox29 and *C. saccharobutylicum* NCP262 showed one mis-

match, and the distances between P1 and P2 sequences in both strains were shorter by 6 bp in *adhA*.

The *C. saccharobutylicum* Ox29 *adhA* deduced amino acid sequence showed 98% homology (similarity) with NADPH-dependent alcohol dehydrogenase Adh-1 from *C. saccharobutylicum* NCP262, up to 82% homology with predicted alcohol (butanol) dehydrogenases from *C. beijerinckii*, and, surprisingly, very low homology (46%) with alcohol (butanol) dehydrogenase from *C. saccharoperbutylacetonicum* (Table 4). Aside from this, AdhA was similar (62%) to the alcohol dehydrogenase part of the bifunctional aldehyde–alcohol dehydrogenases AdhE1 and AdhE2 from *C. acetobutylicum*. These results considered together suggest that there is considerable sequence divergence between certain solventogenesis genes found in the solvent-producing clostridia, and that *C. acetobutylicum* is evolutionarily distinguished from the three other solventogenic *Clostridium* species *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum*.

The genes encoding for aldehyde dehydrogenase, 3-hydroxybutyryl dexydrogenase and crotonase from *C. saccharobutylicum* Ox29 were successfully expressed in *E. coli*. In contrast, no activity of the recombinant butyryl-CoA dehydrogenase could be detected in the cell extract. Reduction of crotonyl-CoA to butyryl-CoA via NAD-independent butyryl-CoA dehydrogenase required an additional electron transferring flavoprotein (EtfAB), which is able to accept electrons from NADH [7]. The assay for Bcd activity in *E. coli* was not successful, probably because of an incomplete or misbalanced chain of cofactors in the heterologous host, as well as the complicated oligomeric structure of the native protein and oxygen sensitivity [5]. Earlier, an NAD-independent butyryl-CoA dehydrogenase activity was successfully measured in cell-free extract of the closely related strain *C. acetobutylicum* NCP262 [11], and the protein was partially (because of protein instability) purified.

The attempts to detect *C. saccharobutylicum* Ox29 AdhA activity (expressed by its own promoter alone and together with the *bcs*-operon) in *E. coli* have failed. In contrast, the *adh-1* gene from *C. saccharobutylicum* NCP262 had been cloned and expressed in *E. coli* under its own promoter. The NADPH-dependent alcohol dehydrogenase Adh-1 was shown to exhibit activity on both butanol and ethanol [28]. The reason for inactivity of the recombinant alcohol dehydrogenase AdhA in *E. coli* is, probably, the low expression level of *adhA*, arising due to disruption of the primary structure of the *adhA* gene promoter region, which has nucleotide replacements and deletions in comparison to the *adh-1* gene promoter region.

The isolation of strain *C. saccharobutylicum* Ox29 shows that the screening of butanologenic clostridia is a promising way of identifying novel bacterial strains with

great potential for the degradation of novel substrates, such as the polysaccharides in plant biomass. Although the strain is not able to degrade or grow on native cellulose, it has high hemicellulolytic activity with extracellular hydrolases and is able to utilize the degradation products. Analysis of its solventogenic genes and their biochemical activity measurement revealed certain differences, especially in the presence of alcohol dehydrogenases, between the four solventogenic clostridial species. This analysis provides further tools for the reconstruction of a synthetic set of 4-carbon solvent-producing genes, which will be of great utility for future biotechnology.

Acknowledgments

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