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New solvent-producing *Clostridium* sp. strains, hydrolyzing a wide range of polysaccharides, are closely related to *Clostridium butyricum*

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Thirteen new *Clostridium* strains, previously isolated from soil and found to produce high amounts of solvents from glucose, hydrolyzed a great variety of α- and β-glycans, including raw starch, xylan, pectin, inulin and cellulose. The sequences of the PCR-amplified DNA fragments containing the variable 3′ part of one of the 16S rRNA genes were 99.5% identical. The macrorestriction pattern of two endonucleolytic digests of chromosomal DNA in the pulsed-field gel electrophoresis (PFGE) confirmed their high homogeneity on the DNA level. The complete 16S rRNA gene sequence of three selected strains was 99.8% identical to the 16S rRNA gene sequence from *Clostridium butyricum* and separates them from *C. acetobutylicum*. To the closely related four species of solventogenic clostridia a new group of strains has to be added, which has a great potential for the direct fermentation of biomass. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 329–335.

Keywords: *Clostridium butyricum*; new strains; solvent production; 16S rRNA sequence; PFGE

Introduction

The biotechnological production of butanol, ethanol and acetone (ABE fermentation) was developed during the First World War. Due to the increasing cost of the substrates sugar cane and beet sugar, following the 1950s the bacterial production of solvents was gradually taken over by petrochemical processes. However, compared to the chemical production, bacterial fermentation of butanol could be cost effective again, when a strain is available that has a high yield in solvent production, selectively produces butanol, and has hydrolytic activity for directly fermenting a wide range of polymeric carbohydrates of low value, such as agroindustrial waste or other biomass. Some saccharolytic, obligately anaerobic bacteria, the acidogenic and solventogenic clostridia, are well suited for this process [5,7].

Although many strains have been screened and successfully used in the industrial fermentation of starch and molasses, only a limited number of them has survived in strain collections [12]. Four distinct groups were identified within the solvent-producing clostridia and characterized as the species *Clostridium acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* [10,13,23]. These species are closely related to each other and belong to group I of the clostridia [2]. Recently a strain of *C. beijerinckii* was used again for development of an economically feasible two-stage solvent-fermentation process from cheap waste biomass [18].

Another species of clostridia group I is *C. butyricum*, which contains saccharolytic bacteria producing mainly butyric acid from glucose and in addition acetic acid, carbon dioxide and hydrogen gas [21]. *C. butyricum* is the dominating anaerobic spore-forming bacterium in certain silages and in spoiled milk products. It ferments a great variety of carbohydrates, including polysaccharides like starch and pectin [8]. Some strains also produce solvents in considerable amounts [1], indicating a possibly similar metabolism as in *C. beijerinckii* and its relatives. The production of 1,3-propanediol from glycerol besides butyric and acetic acids by a newly isolated strain was reported [20]. Environmental factors like substrate, medium composition or growth conditions can greatly change the composition of the fermentation end products [4].

Attempts were made for many years to classify solventogenic clostridia, using different methods including membrane fatty acid characterization, carbohydrate composition, biochemical and physiological tests. To date, 16S rRNA gene sequencing is the most important [13]. 16S rRNA sequence homology can correlate from the level of domain (above 55% homology) to the level of moderately related species (up to 97.5%) [24]. Despite the ease and speed of molecular biological methods (e.g., DNA sequencing), other methods like the RFLP of macrorestriction fragments in the pulsed-field gel electrophoresis (PFGE) have to be used for strain differentiation [13,22].

We reported the isolation of native bacterial strains from Colombia and their preliminary physiological characterization as clostridia [17]. In this study the 13 best solvent-producing strains were investigated by 16S ribosomal gene sequencing and PFGE of macrorestricted chromosomal DNA. However, in contrast to the attribution to three of the four solventogenic species described in that paper, we present data that they are closely related to the species *C. butyricum*, but differ from it with unusual physiological features, like high solvent production. In addition we show that they are extremely homogenous on the DNA level. Due to their growth on a wide range of

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polysaccharides they have the potential for the direct fermentation of biomass into solvents.

Material and methods

Strains and media

The bacterial strains used in this study are listed in Table 1. Reinforced clostridia medium (RCM, Merck) was used to maintain the strains. TY medium was prepared from hydrolyzed casein (16 g), yeast extract (5 g), sodium chloride (5 g), and cysteine (0.5 g per 1000 ml of deionized water). TYG medium is TY with glucose (5 g). Other carbohydrates as substrate were added at 5 g/l if not indicated otherwise. Liquid media were filled in glass vials, flushed with nitrogen, sealed with rubber septa and aluminum crimps. Media were complemented with resazurin (0.05 mg ml^{-1}), and autoclaved under nitrogen for 15 min at 121°C. Anaerobic conditions for agar plates were obtained in an anaerobic chamber with a high purity-grade gas mixture of 85% N_2, 10% H_2 and 5% CO_2. A palladium wafer was used as catalyst. Biomass was determined by extinction at 590 nm.

All cultures were grown from freshly activated *Clostridium* spores: incubation for 10 min at 70°C in the medium indicated.

Hydrolytic activity of culture supernatants was measured from freshly grown 24-h cultures in TYS medium (TY with 1% starch for glucose) induced from spores. Cultures were centrifuged and aliquots of the supernatant were incubated with 0.5% (w/v) of substrates and 0.1 M potassium phosphate buffer (pH 5.6) at 37°C. Reducing sugars liberated from polysaccharides were determined by the dinitrosalicylic acid method [25]. Liberation of glucose from oligosaccharides was measured with the peroxidase–glucose–oxidase assay (PGO; Boehringer Mannheim) as recommended by the manufacturer.

Physiological tests

Freshly grown cultures were streaked on agar plates (TY medium containing 1.5% agar (w/v) and 0.5% carbohydrate) under nitrogen atmosphere at 37°C. Growth in anaerobic liquid cultures (TY medium+0.5% carbohydrate) was assayed by increase in optical density and gas production. Crystalline cellulose and xylan were added (1% w/v). Production of catalase and indole, and the liquefaction of gelatin were assayed as described in Lennette *et al* [14].

Chromosomal DNA preparation

Chromosomal DNA for PFGE was prepared in agarose plugs by a modification of the procedure described in the PFGE Manual, Bio-Rad Laboratories, Germany [22]. Bacteria were grown to an optical density (λ 550 nm) of 0.300 to 0.350 in 40 ml TYG medium. Four DNA plugs (50 μl per plug) were placed in a polyethylene tube with 200 μl EC lysis buffer (6 mM Tris–HCl, 1 M NaCl, 100 mM EDTA, pH 8.0, 0.5% (v/v) Triton X100, 0.5% (w/v) *N*-laurylsarcosine, 1 mg lysozyme and 20 μg RNase A per milliliter). The proteinase K treatment was repeated when necessary. All steps were carried out in the presence of oxygen. For strains degrading the DNA extensively, the procedure was modified: 3.3 U (10 μl) *Thermus* rT41A proteinase (PRETAQ: Gibco BRL) at 56°C was applied for 2 h. The plugs were washed with EET buffer (10 mM Tris–HCl, 100 mM EDTA, 10 mM EGTA; pH 8.0) to inactivate the PRETAQ enzyme.

Table 1 Bacterial strains and their origin

Designation	Species	Origin	
ATCC 824	*C. acetobutylicum*	M. Young	
DSM 1732	*C. acetobutylicum*	DSM	
DSM 792	*C. acetobutylicum*	DSM	
DSM 791	*C. beijerinckii*	DSM	
NCIMB 8052	*C. beijerinckii*	NCIMB	
NRRL B643	*C. saccharobutylicum*	NRRL	
N 1–4	*C. saccharoperbutylacetonicum*	M. Young	
DSM 2478	*C. butyricum*	DSM	
DSM 523	*C. kainantoi*	DSM	
		Department in Colombia	Type of crop
IBUN 13A	this study	Cundinamarca	potatoes
IBUN 18A	this study	Antioquia	chrysantheme
IBUN 18Q	this study	Antioquia	chrysantheme
IBUN 18S	this study	Antioquia	chrysantheme
IBUN 22A	this study	Cundinamarca	potatoes
IBUN 62B	this study	Cundinamarca	grass
IBUN 62F	this study	Cundinamarca	grass
IBUN 64A	this study	Cundinamarca	grass
IBUN 95B	this study	Tolima	soya
IBUN 125C	this study	Tolima	soya
IBUN 137K	this study	Boyaca	sugar cane
IBUN 140B	this study	Boyaca	sugar cane
IBUN 158B	this study	Cundinamarca	tomato

For the new isolates described in this study, the department in Colombia and the type of crop from which soil samples were collected are indicated.

Chromosomal DNA for PCR cloning was extracted from freshly grown cells with the Genomic DNA Kit (Qiagen) as described in the manufacturers booklet. For large-scale isolation of chromosomal DNA, the bacteria were grown in 40 ml RCM for 24 h at 37°C. DNA was isolated by a modified procedure according to Marmur [11]. The DNA was isolated with a glass rod, treated with RNase A (1 mg/ml) and extracted twice with phenol–chloroform (1:1).

Amplification of specific DNA fragments in PCR

All PCR reactions were carried out in a Hybaid Omn E Thermocycler with 25 cycles using 0.3 μM of the respective oligonucleotide primers, 1.25 U *Taq* polymerase (Promega) and 100 ng genomic DNA. PCR products were verified with agarose gel electrophoresis. Each sample was amplified independently three times. PCR products were purified using a Concert Rapid PCR kit (Gibco BRL). The samples were quantified photometrically at 260 nm. Amplified fragments were cloned using the TOPO TA cloning kit (Invitrogen).

The primers of Keis *et al* [13] were modified to optimize the hybridization temperature: for amplification of the complete 16S rRNA gene (5′→3′) pE (gagagtttgagcctggctc), pJ (ttctcctacggctaccttgttac); for amplification of the partial rRNA sequence pA (ggagcaaacaggattagataccc) and pJ; for sequencing the internal primers pC (gtgtcgtgagatgttgg), pD (taacccaacatctcacg), pF (gttttaatcttgcgaccgtac), pG (gtcttcagggacgataatg) and pH (ctgctggcacgtagttag).

DNA sequencing

The DNA sequence of cloned 16S rRNA was determined from supercoiled double-stranded plasmid DNA on both strands with biotinylated oligonucleotide primers (Thermosequenase Cycle Sequencing Kit, Amersham). DNA fragments were separated with a GATC 1500 direct-blotting electrophoresis apparatus (GATC, Konstanz) and visualized with streptavidin-conjugated alkaline phosphatase and the chromogenic substrate NBT-BCIP (Promega).

Sequence data were analyzed and compared with the DNASIS/PROSIS for Windows software package (Hitachi Software Engineering) to the most related 16S rRNA sequences obtained from a BLAST search at EMBL (URL address: http://www.ebi.ac.uk). Sequence similarity analysis was done with the PHYLIP program package obtained from E-mail: http://evolution.genetics.washington.edu/phylip.html. A consensus tree was constructed from using parsimony and likelihood methods [6].

Pulsed-field gel electrophoresis (PFGE)

Agarose plugs containing intact chromosomal DNA (50 μl) were equilibrated at room temperature for 15 min in 100 μl of the restriction enzyme buffer recommended by the manufacturer before preincubation at 4°C for 6 h with 10 U restriction enzyme per plug. The plugs were then digested at the optimal temperature for 12 h. DNA fragments were separated in 1% (wt/vol) multipurpose agarose (Gibco BRL) gels in 0.5 × TBE buffer at 12°C by using contour clamped homogenous field electrophoresis (CHEF System: Gene Navigator System TM, Pharmacia LKB, Uppsala, Sweden). A multistage CHEF program was used to separate the DNA fragments: 170 V constant voltage for 24 h with increasing pulse time of 3, 10, 20, 45, 65, and 90 s (4 h each). Gels were stained with ethidium bromide and viewed with a UV transilluminator.

Substrates

Carboxymethyl cellulose (low viscosity), Avicel (microcrystalline cellulose CF11), xylan (oat spelt and larch wood), soluble starch (potato), pullulan, inulin, pectin, arabinan, chitosan, maltose, cellobiose, raffinose, ribose, sucrose, glycerol and melezitose were obtained from Sigma-Aldrich, raw starch (tapioca starch) from a local food market, and barley β-glucan from Megazyme. Polyosen, the hemicellulose fraction from the Organosolv process of pulp production from spruce wood, was kindly provided by MD Organocell (Germany).

Sequence accession numbers

The GenBank accession numbers for the nucleotide sequences referred to in this paper are: IBUN 22A (acc. no. AJ289704), IBUN 64A (AJ289706) and IBUN 125C (AJ289705).

Results

From soil of agricultural areas in climatically different regions of Colombia planted with various crops, 178 bacteria were isolated as described previously [17]. Thirteen of these mesophilic, anaerobic, spore-forming and acetone-producing new bacteria were selected for further studies due to their superior production of solvents from glucose, which surpassed the production values of the Weizmann strain assayed in parallel (*C. acetobutylicum* ATCC 824). As shown in Table 1, these 13 strains originated from four departments of Colombia and from fields planted with six different crops, representing the outstanding variety of environments in that country [19].

Solvent production reached up to 29.1 g l^{-1} in strain 18A, corresponding to 0.96 mole of solvents produced per mole of glucose fermented, which is close to the theoretical maximum of solvent production [17]. Butyric acid was formed from 2.9 (IBUN 18S) to 5.2 g/l (IBUN 137 K) in an acetate/butyrate ratio from 0.98 to 0.62 (data not shown). The pH of many batch cultures at the end of fermentation was well below pH 5.0, but above pH 6.0 with the cultures most efficiently fermenting alcohols. Under slightly varied conditions, ''acid crash'' [15] was obtained with a medium pH of 4.2 to 4.6 at the end of fermentation (55 h), and acetic acid and especially high amounts of butyric acid were formed (data not shown).

To investigate the potential of the new strains to use biomass as fermentation substrate, carbohydrate utilization was assayed by two methods: growth on an oligo- or polymeric carbohydrate as carbon source and hydrolytic activity in the culture supernatant. In liquid cultures all strains grew under development of gas with glycerol, raffinose, ribose, sucrose and cellobiose. All strains formed colonies on TY-agar containing glucose, cellobiose or soluble starch, but not on xylan, barley β-glucan, carboxymethyl cellulose and crystalline cellulose (Avicel). However, moderate to high hydrolytic activities for these and a number of other polysaccharides were found in the culture supernatants (Table 2). Extracellular enzymes were formed, which released reducing sugars from soluble and raw starch, xylan and carboxymethyl cellulose; by all but one or two strains from pullulan, inulin, pectin and arabinan; and by the majority of the strains from barley β-glucan and chitosan.

Table 2 Relative enzymatic activity in the culture supernatant of fully grown bacterial cultures

Substrate	Strain															
	13A	18A	18Q	18S	22A	62B	62F	64A	95B	125C	137K	140B	158B	Max (U/mg)	N1-4	ATCC 824
Starch	+++	++	++	+	+++	++	++	++	+	+++	++	++++	++	65.0	–	+
Raw starch	+	+++	++	+++	++	+	+	+++	+++	++++	+	++++	+	7.2	+	+
Maltose	–	–	–	–	++	++++	++++	–	–	–	++	–	–	0.3	+++	–
Pullulan	+	++	+++	–	++++	++	++	+	+	++	+	+	+	1.8	++	++
Inulin	+	+	++++	+	++++	+++	++	–	+	+	+	+++	+++	2.4	+	–
Xylan larchwood	+	+	++	+	++++	+++	+++	+	+	++	++	+	++	5.2	–	++++
Xylan oat spelt	++	+	++++	+	+	+++	+++	++	++	+	++	+++	+++	2.8	+	+
Polyosen	+	+	++	++	–	++	++++	++	+/–	++	+/–	+++	–	1.5	–	–
Arabinan	+	+	+	–	++++	+	+	+	–	++	+/–	++	+++	7.4	–	+
Chitosan	+	+	–	–	++++	+++	–	++	+	++	–	++	++	3.3	–	+
Pectin	+	+	++	+	++++	+	+	++	–	++	+	+++	+	6.2	–	+
β-Glucan	–	+++	++++	–	–	++	++	++	++	++	++	++++	++	1.4	–	++++
CMC	+	++	++	+	+++	+	+++	+	+	+	+	++	++++	3.5	–	–
Avicel	+	+	+	+	++++	+	++	++	–	–	++	++	–	4.6	–	++
Cellobiose	–	–	–	++	+	++	++	–	++	–	++	++++	++	0.5	+++	–
Raffinose	+	+	+	+/–	++++	+	++	+	+/–	+	+++	++	+	4.5	–	+
Melezitose	–	+	+++	–	++	++	++++	++	+	+++	++	++	+++	1.3	++	++
Sucrose	+/–	+	++	+/–	++++	++	+/–	+	–	+	+	++	++	2.4	++	++

New strains are indicated by their strain numbers, omitting the IBUN designation. ++++, highest activity of all strains (100%); +++, 75% of maximum activity; ++, 50%; +, 25%; +/–, low activity; –, no activity detected. Activities were determined as glucose equivalent (U min^{-1} mg^{-1} protein).

Surprisingly, hydrolytic activity for maltose and cellobiose was low or not measurable in culture supernatants of most of the strains, although they formed colonies on starch and cellobiose.

To test if just more strains of the known solventogenic clostridia had been isolated, physiological criteria are not sufficient. However, partial 16S rRNA sequencing provides the appropriate tool for classification and has been used to construct a phylogenetic tree of the clostridia [2,13]. Two oligonucleotide primers, pA and pJ, were used to amplify a 688-bp fragment from chromosomal DNA of all new strains containing the most variable downstream part of the small subunit ribosomal RNA gene. The sequence of the individual strains differed by 0 (8 strains), 1 (1), 2 (3) or 3 (1) bases (data not shown). The eight identical sequences were compared to the 16S rRNA sequences in GenBank database: no mismatches were detected with the sequences of the *C. butyricum* strains DSM 2478, ATCC 43755

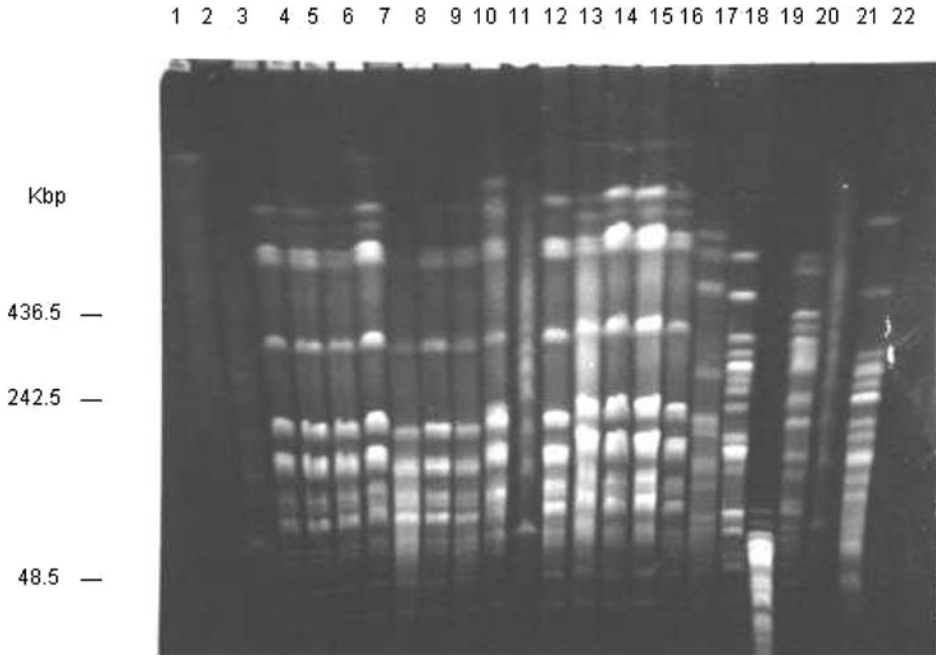

Figure 1 DNA fingerprint of *Sma*I-digested chromosomal DNA of solvent-producing clostridia and the new strains. Lane 2, 11 and 21: λ DNA ladder; 1: undigested control; 3: IBUN 13A; 4: IBUN 18A; 5: IBUN 18S; 6: IBUN 18Q; 7: IBUN 22A; 8: IBUN 62B; 9: IBUN 62F; 10: IBUN 64A; 12: IBUN 95B; 13: IBUN 125C; 14: IBUN 137K; 15: IBUN 140B; 16: IBUN 158B; 17: DMS 2478 (*C. butyricum*); 18: DSM 1732 (*C. acetobutylicum*); 19: NRRL B643 (*C. saccharobutylicum*); 20: NCIMB 8052 (*C. beijerinckii*); 22: N1-4 (*C. saccharoperbutylacetonicum*).

Table 3 Homology of complete 16 S rRNA sequences (1451 bp)

	IBUN 22A	IBUN 64A	IBUN 125C	DSM 2478	DSM 523	NCP 262	DSM 791	ATCC 824
IBUN 22A	100%	6	7	5	7	37	41	129
IBUN 64A	99.6%	100%	5	3	5	36	37	131
IBUN 125C	99.5%	99.7%	100%	2	4	36	36	133
DSM 2478	99.7%	99.8%	99.9%	100%	2	36	35	132
DSM 523	99.5%	99.7%	99.7%	99.9%	100%	36	34	134
NCP 262	97.3%	97.4%	97.4%	97.5%	97.4%	100%	18	106
DSM 791	96.9%	97.5%	97.4%	97.7%	97.2%	98.6%	100%	138
ATCC 824	90.3%	90.2%	90.3%	90.2%	90.4%	92.0%	89.7%	100%

Lower half: percentage of identity within 1432 bases. Upper half: number of mismatches between two sequences.

and NCIMB 8082. In contrast, the 16 S rRNA sequence of *C. saccharoperbutylacetonicum* (strain N1-4), the next most related in a CLUSTAL alignment, differed by 17 residues in that region (97.5% identity).

The similarity between the new strains, as shown by the partial 16S rRNA sequences, was corroborated on the DNA level by digesting the chromosomal DNA with the rare cutting restrictions enzymes *Sma*I and *Apa*I, selected from restrictases with 6-bp G+C-rich recognition sites (Figure 1). The pattern of the ca. 15 *Sma*I and ca. 19 *Apa*I fragments from about 20 to over 600 kb was indistinguishable, except for 1 (2) additional *Sma*I (*Apa*I) fragments of large size for strain IBUN 64A (data for *Apa*I not shown). This indicates a high degree of similarity in DNA sequence as well as in gross chromosomal structure and arrangement. Very few bands of equal size could be identified in the pattern of chromosomal DNA fragments from the solventogenic group I clostridia DSM 1732, NRRL B643, NCIMB 8052 and N1-4, but also fro DSM 2478 (*C. butyricum*). These PFGE data corroborated the sequence data in that all strains were almost identical to each other, but different from the type strains of the group I solventogenic clostridia. They showed also considerable differences with the chromosomal DNA of *C. butyricum*.

The homogeneity of the new strains on the DNA level allowed for reducing the number of further investigations. To provide a broader data basis, the sequence of the complete gene for the small ribosomal RNA was obtained for the strains with the highest fermentation potential or the highest hydrolytic enzyme activities: IBUN 22A (acc. no. AJ289704), IBUN 64A (AJ289706) and IBUN 125C (AJ289705). These sequences include 1451 bases and correspond to the *E. coli* 16S rRNA numbering 7 to 1496. They were compared in a BLAST search to the EMBL data base. The most similar sequences (99.5–99.9%) were the 16S rRNA sequences from *C. butyricum* type strain DSM 2478^T and the closely related bacterium *C. kainantoi* (DSM 523) (Table 3). The homology with sequences from *C. saccharobutylicum* and

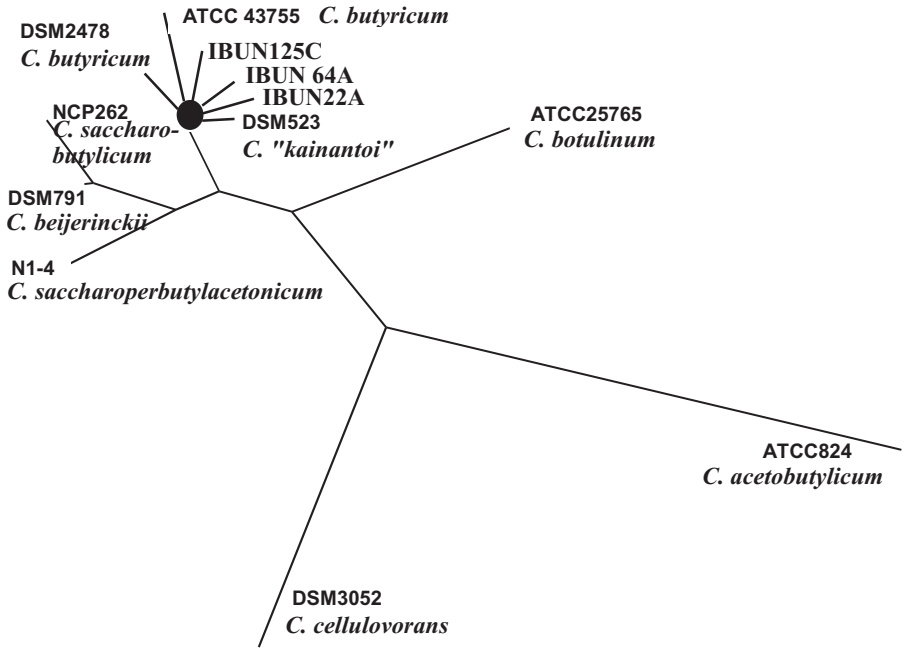

Figure 2 Similarity tree of complete 16S rRNA sequences of the most related group I clostridia including three of the new strains. The tree was constructed with the PHYLIP program package. The cluster of sequences with up to seven mismatches is not resolved.

C. beijerinckii with 96.9% to 97.5% and from *C. acetobutylicum* with 90.2% identity was lower. The high degree of identity of more than 99.5% over 1451 bases with *C. butyricum* does not allow further subclassification. A similarity tree was constructed using the CLUSTAL method (Figure 2).

Discussion

The new solvent-producing bacteria were isolated from cultured land with different crops in four departments of Colombia, which represent four climate zones of the valleys of central Colombia (Table 1). Even under nonoptimized fermentation conditions solvent production reached the maximum of solvents obtained with the best industrial strains [5,17]. Some strains were tolerant of high concentrations of the toxic solvents produced. The increase of pH at the end of the fermentation and the occasional acid crash indicated that the acids were produced in a first acidogenic stage of the fermentation and were then converted to alcohols in a second, solventogenic stage. Therefore, it can be expected that the new strains possess genes for a solvent-production pathway analogous to *C. acetobutylicum* [3]. The solvent production could be further improved by gene engineering or by changing the process parameters to optimize conversion of carbohydrates into alcohols, the desired end product [5,23].

Solvent production with the new strains has been performed with glucose as substrate [17]. However, glucose is a relatively expensive fermentation substrate usually obtained from starch by enzymatic hydrolysis. It would be advantageous if the solventogenic strains could make use of the polymeric biomass carbohydrates without pretreatment. Therefore, the new strains have been tested for their hydrolytic activities. A wide range of polysaccharides occurring in biomass was hydrolyzed containing 1,2-, 1,3-, 1,4-, 1,5-, 1,6-linkages of α-, and 1,2-, 1,3-, 1,4-linkages of β-glycans. The highest overall activity was found in the culture liquid of strain IBUN 22A. Comparatively low activities were observed in culture fluids of all strains for hydrolysis of the disaccharides tested, which the cells presumably take up and degrade intracellularly. Strains IBUN 18Q, 62B, 62F and 140B also have outstanding polysaccharide hydrolyzing activities, making them good candidates for direct fermentation of biomass.

The new strains were characterized as Gram-positive, spore-forming, mesophilic, strictly anaerobic rods. They did not form catalase, gelatin hydrolyzing protease, and indole; they acidified the medium on growth with glucose, ribose, starch, sucrose and raffinose, hydrolyzed cellobiose (β-glucosidase) and grew on glycerol. In addition to solvents, acetic and butyric acids were produced from glucose. At least strain IBUN 64A was able to grow in TY medium without any additional carbohydrate. The strains clearly belong to the saccharolytic clostridia (group I) [1]. However, they did not match, as could be expected, the physiological pattern described for the solventogenic species *C. acetobutylicum, C. beijerinckii, C. saccharobutylicum* and *C. saccharoperbutylacetonicum*, but behaved like *C. butyricum*.

This surprising result was confirmed by the analysis of the 16S rRNA sequences, which placed the newly isolated clostridia as a homogeneous group near to *C. butyricum* and *C. kainantoi* and clearly separated them from other solventogenic (like *C. acetobutylicum*) or saccharolytic group I clostridia like *C. botulinum* or *C. cellulovorans* (Figure 2). These data thus supported the results obtained with the physiological characterization. The level of 0.3% sequence mismatches with the 16S rRNA gene of *C. butyricum* does not qualify to create a new species. But the physiological trait of high solvent production distinguishes the new strains from *C. butyricum* and would deserve the creation of a new subspecies of *C. butyricum*, which would have to be established in a separate investigation.

To differentiate bacterial strains within a species or between closely related species, the determination of the macro-restriction pattern of chromosomal DNA fragments in the PFGE is a successful method within clostridia [13]. As shown for four *C. stercorarium* strains, the restriction pattern within a species may vary considerably [22]. Despite isolation from different locations and their variability in fermentation and hydrolytic activities, the fragment pattern of two restriction endonuclease digests showed almost no variation within the newly isolated strains (Figure 1); but their pattern was clearly different from any of the *Clostridium* group I strains applied, including *C. butyricum*. This excludes a very close relationship at least with the type strain DSM 2478. Nevertheless, the production of type E botulism toxin, described to occur in some soil-related *C. butyricum* strains [9,16], should be checked with all strains.

The new clostridia thus form an extremely homogeneous group of strains clearly different from the solventogenic clostridia. No member of the well-known four solventogenic clostridial species was among the best 13 solvent producers. This might be a result of the tight screening regime (spore formation and reactivation conditions; rich medium; fermentation conditions) that could exclude other solventogenic clostridia. It would have to be evaluated if the other solventogenic *Clostridium* species were also present in soil samples from Colombia, as saccharolytic, mesophilic clostridia are generally regarded as ubiquitous.

Although the homogeneity on the DNA level of the new strains is startling, similar results have been described for the four solventogenic clostridial species [13], despite the fact that some strains of one species were isolated from different continents or were propagated in separate strain collections for up to 80 years. This could reflect an extraordinary genetic stability within species of the group I of the clostridia (D.T. Jones, personal communication).

Discovery of these new *C. butyricum* strains adds considerably to the potential for industrial solvent fermentation by combining the hydrolytic abilities of *C. butyricum* with the solvent-producing abilities of the solventogenic clostridia. The broader evolutionary basis could help to solve problems with the classical solvent fermentation, such as strain degeneration, sensitivity to bacteriophages, regulation of the acidogenic–solventogenic switch, or limitations in the product range and in the accessibility of cheap substrates. The new *C. butyricum* strains could therefor substantially contribute to the revival of biotechnological solvent production and challenge the petrochemical production of solvents.

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