Taxonomy and Phylogeny of Industrial Solvent-Producing Clostridia

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We performed a systematic study of 55 solvent-producing clostridial strains, the majority of which are currently classified as *Clostridium acetobutylicum* strains, by using a combination of biotyping and DNA fingerprint analysis. The biotyping procedures used included rifampin susceptibility testing, bacteriocin typing, and bacteriophage typing. The 55 strains examined exhibited a good correlation between their biotypes and DNA fingerprints, which allowed us to divide them into nine groups. The DNA fingerprints of the nine groups differed markedly, but within each group the DNA fingerprints exhibited a high level of similarity. To determine the phylogenetic relationships of the nine groups, we performed a 16S rRNA gene sequence analysis. The results of a comparative analysis of the partial sequence corresponding to positions 830 to 1383 (*Escherichia coli* numbering) of the 16S rRNA gene indicated that the nine biotype groups could be assembled into four taxonomic groups. The complete 16S rRNA sequences of strains representing these groups were determined. Our phylogenetic analysis revealed that the amylolytic type strain *C. acetobutylicum* ATCC 824 (taxonomic group I) was only distantly related to the saccharolytic strains belonging to taxonomic groups II, III, and IV (levels of sequence similarity, 90 to 90.5%). The strains belonging to taxonomic groups II, III, and IV, represented by *C. acetobutylicum* NCP 262, "*Clostridium saccharoperbutylacetonicum*" N1-4, and *C. acetobutylicum* NCIMB 8052^T (T = type strain), respectively, were closely related (levels of sequence similarity, 98.2 to 98.9%). *C. acetobutylicum* NCIMB 8052^T exhibited a level of similarity of 100% with the type strain of *Clostridium beijerinckii*. Reclassification of the saccharolytic solvent-producing strains is necessary, and possible names for the four taxonomic groups are discussed.

Over the last few years workers have made progress in elucidating the complex phylogenetic relationships which exist among members of the large, heterogeneous group of gram-positive, anaerobic, spore-forming bacteria presently grouped together in the genus *Clostridium* (5, 6, 18, 23). Advances have been made largely through the use of 16S rRNA oligonucleotide cataloging and gene sequencing (5, 6). More than 100 clostridial 16S rRNA gene sequences have now been determined, and the resulting data have provided the basis for a taxonomic revision of the clostridia (6). The results of recent studies have indicated that the clostridia and their close relatives represent several divergent and deeply branched lineages (5, 6). The largest and phylogenetically most well-defined lineage is the lineage that was designated rRNA homology group I by Johnson and Francis (18) and was referred to by Collins et al. as cluster I (6). Included in homology group I is the type species of the genus, *Clostridium butyricum*, along with many of the common mesophilic, butyric acid-producing clostridial species. Also included in this homology group are *Clostridium acetobutylicum*, *Clostridium aurantibutyricum*, and *Clostridium beijerinckii*, species that are capable of producing significant amounts of butanol and other neutral solvents, such as acetone, isopropanol, and ethanol, during the late stages of growth in batch fermentation cultures (21).

Industrial production of acetone and butanol by solvent-producing *Clostridium* spp. strains was one of the first large-scale fermentation processes developed (20). The first successful industrial starch-fermenting clostridial strain was isolated by Weizmann (44), and this strain was used extensively to produce acetone from maize mash during World War I. After this war an American company, Commercial Solvents Corporation (CSC), acquired the acetone-butanol (AB) fermenta-

tion plants in the United States and the patent rights for the Weizmann process (20). With the expiry of the Weizmann patent in 1935, the AB fermentation process became available worldwide, and this process continued to be the major supplier of industrial solvents until it was superseded in most countries by chemical synthesis in the 1950s and 1960s (20). However, the AB fermentation process was used in South Africa until the early 1980s and is still used in the People's Republic of China.

Although maize mash was initially used as the fermentation substrate, after the mid-1930s most fermentation processes were converted to processes in which molasses was used (10, 38). However, attempts to use the starch-fermenting strains of clostridia in these processes were unsuccessful, even when invert molasses was used (30). As a result, a succession of new saccharolytic, solvent-producing strains with different phenotypic characteristics were isolated, developed, and patented by the various companies engaged in the production of acetone and butanol (10, 30, 38). For the purpose of obtaining a patent, each new strain was assigned a novel species name, but the nomenclature adopted was not systematic and was applied in a haphazard manner (38, 39). Scientifically, most of the strains were never recognized as legitimate species, and after the industrial fermentation process went into decline, these names were no longer used. The origins and development of the various patented strains have been discussed in a recent review by Jones and Keis (19). At present the majority of the industrial solvent-producing strains tend to be classified as *C. acetobutylicum* strains. Despite the important role that the solvent-producing clostridia played in industrial microbiology, there have been few taxonomic studies of this group of bacteria.

In 1926 McCoy et al. performed a comparative study of 11 starch-utilizing strains (Table 1) (26). These workers concluded that the small phenotypic variations which occurred in the 11 strains did not justify separating these organisms into

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TABLE 1. *C. acetobutylicum* strains included in early comparative studies

Authors	Year	Reference	Source and/or strain designation	Code ^a	Year isolated	Origin
McCoy et al.	1926	26	CSC, Weizmann strain	50	1915	Corn
			CSC	55	1920	Molasses
			CSC	60	1923	Corn mash
			CSC, BF strain	65	1918	Unknown (used at Melle, France)
			CSC, Pike-Smythe strain	70	1922	Unknown
			Carroll strain (Wisconsin)	100	1923	Garden soil
			CSC	105	1919	Barley
			Fred strain (Wisconsin)	150	1920	Lake Mendota mud
			CSC, Speakman strain	180	1923	Barley (repurified)
			Carroll strain (Wisconsin)	200	1923	Field soil
			Mulvania strain (Wisconsin)	300	1923	Potato
Weyer and Rettger	1927	45	Weizmann strain?	S		Unknown (from another laboratory)
			CSC strain	St	1919	Barley (original solvent culture)
			Weyer/Rettger	So	1924	Connecticut garden soil (= ATCC 824 ^T)
			Weyer/Rettger	K	1924	Putrefying clam
			Weyer/Rettger	M	1924	Corn meal
			Weyer/Rettger	B	1925	Barley
McCoy and McClung	1935	27	Weizmann strain (ex Andrewes)	W ₁₇	1914	Original Weizmann culture
			Hall strain no. 462	H		Industrial (= ATCC 3625)
			Thaysen strain (Weizmann strain)	T		Used in war (= ATCC 4259)
			Donker/Kluyver strain	D		Unknown (= ATCC 862)
			Weyer strain	W	1924	Connecticut garden soil (= ATCC 824 ^T)
			Andrewes, X160 strain	A		Unknown
			Andrewes, Bakonyi strain	B		Unknown
			Granulobacter	Gr II	1926	Unknown
			CSC, strain No. 1	C	1931	Industrial
			CSC, strain No. 2	U	1931	Industrial
			McCoy strain (Wisconsin)	M	1931	Corn

^a Original code designation assigned by the authors.

different types and recommended that all 11 strains should be grouped in a single species named *C. acetobutylicum* (26).

A year later, Weyer and Rettger published the results of a similar study in which they compared the phenotypic characteristics of six different starch-utilizing, solvent-producing strains (Table 1) (45). These authors also concluded that all of their strains belonged to a single clearly defined species, and they proposed that this species should be called *C. acetobutylicum* Weizmann. One of these strains, strain ATCC 824, which was isolated from Connecticut garden soil in 1924, was selected by them as the type strain of the species (45). Two other strains included in their study were from unspecified sources, and one of these apparently was derived from the original Weizmann industrial production strain.

In a follow-up study McCoy and McClung (27) investigated the serological relationships of 22 strains of *C. acetobutylicum* by studying serological agglutination reactions. In this survey McCoy and McClung included the original 11 strains used in the study of McCoy et al. (26), as well as an additional 11 strains obtained from various sources (Table 1). The findings of this study again revealed that on the basis of serological test results no subdivisions could be identified within the group of strains tested. These strains were cross-tested with strains belonging to the "*Clostridium butylicum*" and *C. beijerinckii* groups, and no appreciable cross-reactions were observed with any of the strains (27).

In all three studies the authors concluded that the strains which were tested belonged to a closely related assemblage of microorganisms which could be grouped together in a single species. These organisms, typified by the type culture and Weizmann industrial strains, were selected for their ability to produce high solvent yields on starched-based substrates, such as maize. No comparative studies were performed with the

saccharolytic strains isolated and developed for their ability to produce solvents from molasses.

In a taxonomic study performed by Cummins and Johnson (8) strains labeled *C. butyricum* and *C. beijerinckii* were investigated, and the majority of these strains could be assigned to two clearly distinct homology groups. Strains labeled *C. acetobutylicum* did not exhibit a high level of homology with either the *C. butyricum* homology group or the *C. beijerinckii* homology group (8).

In a later study in which DNA-rRNA pairing methods were used, Johnson and Francis identified three major homology groups in the clostridia, and one of these groups, homology group I, was further subdivided into 10 subgroups (subgroups IA through IJ) (18). The *C. butyricum* and *C. beijerinckii* strains were assigned to subgroup IA, whereas *C. acetobutylicum* was assigned to subgroup IJ. In a recent study, however, Collins et al. (7) reported that on the basis of 16S rRNA gene sequence data, the *C. acetobutylicum* type strain, strain NCIMB 8052, exhibited high levels of homology with *C. beijerinckii* strains.

As a result of the extensive research performed over the last decade, it has become apparent that a number of the most commonly studied solvent-producing strains, such as strains ATCC 824^T (T = type strain), DSM 792^T, NCIMB 8052^T, ATCC 4259, NCP 262, N1-4, and ATCC 39058, differ substantially in their physiological and genetic characteristics and that these organisms do not appear to constitute a homologous group. In a recent study Wilkinson and Young found that a number of these strains produced different genomic DNA restriction patterns and that their genome sizes differed (46).

In this study biotyping, DNA fingerprint analysis, and 16S rRNA gene sequencing were used to investigate the taxonomic and phylogenetic relationships of 55 strains of solvent-producing clostridia, including 17 industrial strains and 38 strains

obtained from the American Type Culture Collection (ATCC), Rockville, Md.; DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Göttingen, Germany; National Collection of Industrial and Marine Bacteria (NCIMB), Aberdeen, Scotland; and Northern Utilization Research and Development Division (NRRL), Peoria, Ill.

MATERIALS AND METHODS

Bacterial strains and cultivation. The *C. acetobutylicum*, *C. beijerinckii*, "*Clostridium madisonii*," and "*Clostridium saccharoperbutylacetonicum*" strains used in this study are listed in Table 2. These clostridial strains were maintained as spore stock preparations stored in sterile distilled water at 4°C. Spores were germinated by heat shocking them at 70°C for 2 min and then rapidly cooling them on ice for 45 s before they were inoculated into an appropriate culture medium under anaerobic conditions. The clostridial strains were grown anaerobically at 34°C in *Clostridium* basal medium (CBM) (33) or in tryptone-yeast-glucose medium (13) which was supplemented with 0.01 g of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ per 1,000 ml, 1 mg of *p*-aminobenzoic acid per 1,000 ml, 1 mg of thiamine hydrochloride per 1,000 ml, and 0.2 mg of biotin per 1,000 ml. When necessary, both media were solidified with 1.5% (wt/vol) agar. CBM agar supplemented with 2% (wt/vol) soluble starch and 2% (wt/vol) sucrose was used for the bacteriophage assay and for spore propagation.

Rifampin susceptibility. Filter paper discs impregnated with two concentrations of the antibiotic rifampin (10 and 100 ng per disc) were used routinely. Confluent lawns of each strain were prepared by flooding agar plates with a bacterial culture (optical density at 600 nm, 0.30 to 0.40); the excess liquid was removed, and the surface of the agar was allowed to dry before rifampin discs were placed onto the agar surface. The agar plates were incubated anaerobically at 34°C for 8 to 12 h. A strain was considered susceptible to rifampin if a zone of inhibition surrounded a 10-ng rifampin disc and resistant if no growth inhibition was observed around a 100-ng rifampin disc.

Bacteriocin production and typing. Bacteriocin production in *C. acetobutylicum* NCP 262 was induced in 10-ml CBM broth cultures (optical density at 600 nm, 0.30) by adding mitomycin C (final concentration, 0.5 µg/ml). After 30 min of incubation at 34°C, the cells were pelleted and resuspended in prewarmed (34°C) CBM broth. The culture was then incubated for an additional 2 h at 34°C. The culture supernatant was harvested following centrifugation at $3,200 \times g$ for 15 min, filter sterilized, and stored at 4°C. A second bacteriocin-like inhibitory agent was obtained from an uninduced supernatant from an 18-h *C. acetobutylicum* NCP 258 culture. Bacteriocin activity was assayed by spotting 10-µl portions of the culture supernatants onto bacterial lawns and examining the preparations for growth inhibition after anaerobic incubation at 34°C overnight. Bacteriocin activity was indicated by complete clearing of the area spotted onto the bacterial lawn. Dilution of the culture supernatants onto susceptible strains did not produce individual plaques, indicating that the effect on growth was due to a bacteriocin and not a bacteriophage.

Bacteriophage typing. Eight previously isolated bacteriophages were used for biotyping (Table 2). Bacteriophage CA1 was isolated in 1980 from an abnormal industrial fermentation at National Chemical Products (NCP), Ltd., Germiston, South Africa (37). Bacteriophage CA2 was isolated by workers in the Department of Microbiology at the University of Cape Town, Cape Town, South Africa. Bacteriophages HMT, HM2, HM3, and HM7 originated from infections which occurred in the Japanese fermentation industry and have been described previously (12, 14). Bacteriophage HMT was isolated from "*C. saccharoperbutylacetonicum*" ATCC 27021. Bacteriophage CMX was isolated from "*C. madisonii*" 4J9 in 1948 (35, 36). All bacteriophages were maintained as lysates in phage buffer (10 mM Tris-Cl [pH 7.4], 100 mM NaCl, 10 mM MgSO_4) at 4°C. Bacteriophage lysates (approximately 10^6 PFU/ml) were spotted onto bacterial lawns as described above for bacteriocin typing, and the preparations were examined for the presence of lysis in the bacterial lawns after anaerobic incubation at 34°C overnight. The high concentrations of bacteriophages used in the phage lysates resulted in complete lysis of sensitive host cells where the bacteriophages were applied. Dilution of phage lysates resulted in the presence of individual plaques on sensitive bacterial lawns.

Chromosomal DNA preparation. Chromosomal DNA was prepared by using a modification of the procedure described by Borges and Bergquist (4). Bacteria were grown to an optical density at 600 nm of 0.24 to 0.30 in 10 ml of CBM or tryptone-yeast-glucose medium. The cells were harvested by centrifugation and washed with 10 ml of ET buffer (100 mM EDTA, 10 mM Tris-Cl; pH 8.0) before they were resuspended in 0.5 ml of ET buffer-TE buffer (25 mM Tris-Cl, 10 mM EDTA, 15% [wt/vol] sucrose) (50:50). An equal volume of molten 2% SeaPlaque low-melting-point agarose (FMC BioProducts) in EET buffer (10 mM Tris-Cl, 100 mM EDTA, 10 mM EGTA; pH 8.0) that had been precooled to 42°C was added to the cell suspension, and the mixture was poured into a plug-forming mold. Solidified plugs (250 µl per plug) were incubated overnight at 37°C in an equal volume of lysis buffer (10 mM Tris-Cl [pH 8.0], 1 M NaCl, 0.01 mM EDTA, 0.05% [vol/vol] Triton X-100, 0.2% [wt/vol] deoxycholate, 0.5% [wt/vol] *N*-lauroylsarcosine, 1 mg of lysozyme per ml, 20 µg of RNase A per ml). The plugs were washed twice with reaction buffer (10 mM Tris-Cl [pH 8.0], 1% [wt/vol]

N-lauroylsarcosine, 0.01 mM EDTA) before they were immersed in reaction buffer (1.25 ml per three DNA gel plugs) and 18.75 U (62.5 µl) of *Thermus* rT41A proteinase (PRETAQ; Gibco BRL) was added. All of the steps described above were carried out in an anaerobic glove box (Forma Scientific, Inc.). The plugs and the reaction buffer were sealed in a gas-tight glass vial, transferred out of the glove box, and immediately placed in a 56°C water bath for 2 h. The gel plugs were washed three times with TE buffer (10 mM Tris-Cl, 1 mM EDTA; pH 8.0) (10 ml per three plugs) before they were stored at 4°C in EET buffer. When we encountered problems with the isolation of intact DNA, the procedure described above was modified. In these cases, the cells were harvested and incubated anaerobically in 1 ml of reaction buffer containing PRETAQ at 56°C for 2 h before they were washed with ET buffer.

Restriction enzyme digestion. A number of restriction endonucleases with G+C-rich recognition sequences were tested. These included the 8-base recognition enzymes *Asc*I, *Not*I, *Rsr*II, *Sfi*I, *Sgr*AI, and *Sse*8387I and the 6-base recognition enzymes *Apa*I, *Age*I, *Bgl*II, *Bss*HII, *Eag*I, *Ecl*XI, *Ksp*I, *Mlu*I, *Nae*I, *Nar*I, *Nci*I, *Sma*I, and *Sul*I. In addition, restriction enzymes *Bln*I, *Spe*I, and *Xba*I, which have the rare CTAG tetranucleotide in their recognition sequences (25), were investigated. Slices of DNA plugs (12 to 24 µl) were equilibrated three times at room temperature for 15 min in 100 µl of the restriction enzyme buffer recommended by the manufacturer before preincubation at 4°C for 4 to 16 h with 10 U of restriction enzyme. The plugs were then digested at the optimal temperature for 9 to 16 h.

Pulsed-field gel electrophoresis (PFGE). Digested DNA plugs were equilibrated three times in TAFEII buffer (22.5 mM Tris-borate, 0.5 mM EDTA) at room temperature for 15 min before gels were loaded. DNA fragments were separated on 1% (wt/vol) multipurpose agarose (Boehringer Mannheim) gels in TAFEII buffer at 14°C by using a transverse alternating field electrophoresis system (GeneLine II; Beckman Instruments). Two multistage transverse alternating field electrophoresis programs were used to separate the DNA fragments. The electrophoretic conditions for program 1 were as follows: stage 1, constant current of 250 mA for 5 h with a 4-s pulse; stage 2, constant current of 250 mA for 5 h with an 8-s pulse; stage 3, constant current of 250 mA for 5 h with a 16-s pulse; stage 4, constant current of 300 mA for 5 h with a 24-s pulse; and stage 5, constant current of 300 mA for 5 h with a 32-s pulse. Program 2 consisted of the five stages described above for program 1 followed by a stage 6 (constant current of 350 mA for 5 h with a 60-s pulse) and a stage 7 (constant current of 350 mA for 5 h with a 120-s pulse). Following electrophoresis, the gel was stained in ethidium bromide and destained in deionized water before it was viewed with a UV transilluminator.

Design of oligonucleotide primers. The sequences of the 16S rRNA genes from *C. acetobutylicum* NCIMB 8052^T (7), *C. beijerinckii* DSM 791^T and NCIMB 9362^T, *Clostridium botulinum* (nonproteolytic) types B, E, and F, and *C. butyricum* ATCC 43755, DSM 2478, and NCIMB 8082 (15) were aligned by using the multiple sequence alignment program PILEUP of the Genetics Computer Group, Madison, Wis. (9). On the basis of this alignment we chose a PCR primer pair consisting of primer PCR A (5'-GGAGCAAACAGGATTAGATACC-3'; positions 774 to 795 [*Escherichia coli* numbering system]) and primer PCR B (5'-TGCCAACTCTATGGTGTGACG-3'; positions 1419 to 1403) because they flanked a relatively variable region of the 16S rRNA gene (Fig. 1). Additional PCR primers were designed to amplify the complete 16S rRNA molecule. These included the PCR primer pair consisting of primer PCR E (5'-GAGAGTTT GATCCTGGCTC-3'; positions 7 to 25) and primer PCR F (5'-GAGTTTAT ATCTTGCGACCGTAC-3'; positions 911 to 889), as well as primers PCR I (5'-GCTACCATTTAGTTGAGCACTC-3'; positions 1126 to 1148) and PCR J (5'-TTCTCCTACGGCTACCTTGTTACG-3'; positions 1520 to 1496) (Fig. 1). The sequencing primers used included the PCR primers, as well as primers SEQ C (5'-GTGTCGTGAGATGTTGG-3'; positions 1071 to 1087), SEQ D (5'-TAACCAACATCTCAGC-3'; positions 1091 to 1075), SEQ G (5'-GTCT TCAGGACGACGATATGACGGTAC-3'; positions 437 to 487), and SEQ H (5'-GCTGCTGGCACGTAGTTAGCCG-3'; positions 524 to 503) (Fig. 1). The primer sequences which we chose were based on regions which were highly conserved and optimally spaced for DNA sequencing, and we made sure that each nucleotide was sequenced at least once on each strand.

PCR amplification and purification of 16S rRNA. The regions from position 830 to position 1383 (*E. coli* numbering) of 11 solvent-producing clostridial strains were amplified by using PCR primers PCR A and PCR B (Fig. 1). The complete 16S rRNA genes of four of these strains were amplified as three overlapping fragments that were approximately 650 bp long (by using PCR primers PCR A and PCR B), 870 bp long (by using PCR primers PCR E and PCR F), and 390 bp long (by using PCR primers PCR I and PCR J) (Fig. 1). Agarose-embedded DNA plugs were used as DNA templates for PCR amplification. Plugs which had been stored in EET buffer were washed four times in TE buffer. A slice (25 µl) of an equilibrated agarose plug was heated at 90°C for 20 min to inactivate any traces of PRETAQ, and 10 µl of the molten DNA plug was added to a 100-µl PCR mixture containing each of the appropriate primers at a concentration of 0.2 µM. A *UITma* DNA polymerase PCR kit (Perkin-Elmer Cetus) was used as recommended by the manufacturer along with *AmpliWax* hot start pellets (Perkin-Elmer Cetus). PCRs were performed in a Hybaid OmniGene thermal cycler. The initial amplification cycle consisted of incubation at 92°C for 2 min, at 55°C for 2 min, and at 72°C for 5 min; this was followed by 28 cycles consisting of incubation at 92°C for 30 s, at 55°C for 30 s, and at 72°C for

TABLE 2. Groups of solvent-producing clostridial strains based on biotyping and DNA fingerprint analysis results

Group	Species	Strain	Susceptibility to rifampin ^a	Inhibition by bacteriocins ^b		Cell lysis by bacteriophages ^c					DNA fingerprint ^d
				NCP 258	NCP 262	CA1 and CA2	CMX	HM2 and HMT	HM3	HM7	
1	<i>C. acetobutylicum</i>	ATCC 824 ^T	S	—	—	—	—	—	—	—	1
		= DSM 792 ^T	S	—	—	—	—	—	—	—	1
		= NRRL B527 ^T	S	—	—	—	—	—	—	—	ND
	<i>C. acetobutylicum</i>	ATCC 3625	S	—	—	—	—	—	—	—	1
		= DSM 1737	S	—	—	—	—	—	—	—	1
	<i>C. acetobutylicum</i>	ATCC 4259	S	—	—	—	—	—	—	—	ND
		= DSM 1731	S	—	—	—	—	—	—	—	1a
		= NCIMB 619	S	—	—	—	—	—	—	—	ND
		= NRRL B530	S	—	—	—	—	—	—	—	ND
	<i>C. acetobutylicum</i>	ATCC 8529	S	—	—	—	—	—	—	—	ND ^e
		= DSM 1738	S	—	—	—	—	—	—	—	ND
	<i>C. acetobutylicum</i>	DSM 1732	S	—	—	—	—	—	—	—	ND ^e
		= NCIMB 2951	S	—	—	—	—	—	—	—	ND
	<i>C. acetobutylicum</i>	DSM 1733	S	—	—	—	—	—	—	—	1
	= NCIMB 6441	S	—	—	—	—	—	—	—	ND	
<i>C. acetobutylicum</i>	NCIMB 6442	S	—	—	—	—	—	—	—	1	
<i>C. acetobutylicum</i>	NCIMB 6443	S	—	—	—	—	—	—	—	ND ^e	
<i>C. acetobutylicum</i>	ATCC 39236	S	—	—	—	—	—	—	—	1b	
<i>C. acetobutylicum</i>	ATCC 43084	S	—	—	—	—	—	—	—	ND ^e	
2	<i>C. acetobutylicum</i>	NCP 262	S	—	—	—	—	—	—	—	2
	<i>C. acetobutylicum</i>	NCP 249	S	—	—	—	—	—	—	—	2
	<i>C. acetobutylicum</i>	NCP 265	S	—	—	—	—	—	—	—	2
	<i>C. acetobutylicum</i>	NCP 268	S	—	—	—	—	—	—	—	2
	<i>C. acetobutylicum</i>	NCP 258	S	—	—	—	—	—	—	—	2a
	<i>C. acetobutylicum</i>	NRRL B643	S	—	—	—	—	—	—	—	2a
3	“ <i>C. saccharoperbutylacetonicum</i> ”	N1-4	R	+	+	—	—	+	+	—	3
		= ATCC 13564	R	+	+	—	—	+	+	—	ND
	“ <i>C. saccharoperbutylacetonicum</i> ”	ATCC 27021	R	+	+	—	—	—(L)	+	—	ND
4	“ <i>C. saccharoperbutylacetonicum</i> ”	N1-504	R	—	+	—	—	—	—	+	4
		= ATCC 27022	R	—	+	—	—	—	—	+	ND
5	<i>C. acetobutylicum</i>	NCP 193	R	—	—	+	—	—	—	—	5
	<i>C. acetobutylicum</i>	NCP 172	R	—	—	+	—	—	—	—	5
	<i>C. acetobutylicum</i>	NCP 259	R	—	—	+	—	—	—	—	5
	<i>C. acetobutylicum</i>	NCP 261	R	—	—	+	—	—	—	—	5
	<i>C. acetobutylicum</i>	NCP 263	R	—	—	+	—	—	—	—	5
	<i>C. acetobutylicum</i>	NCP 264	R	—	—	+	—	—	—	—	5
	<i>C. acetobutylicum</i>	NCP 270	R	—	—	+	—	—	—	—	5
	<i>C. acetobutylicum</i>	NCP 271	R	—	—	+	—	—	—	—	5
6	<i>C. acetobutylicum</i>	NCIMB 8052 ^T	R	+	+	—	+	—	—	—	6
	<i>C. acetobutylicum</i>	NCIMB 8049	R	+	+	—	+	—	—	—	6
		= DSM 1739	R	+	+	—	+	—	—	—	6
		= ATCC 10132	R	+	+	—	+	—	—	—	6
		= NRRL B594	R	+	+	—	+	—	—	—	6
	<i>C. acetobutylicum</i>	NCIMB 6444	R	+	+	—	+	—	—	—	ND
	<i>C. acetobutylicum</i>	NCIMB 6445	R	+	+	—	+	—	—	—	6
	<i>C. acetobutylicum</i>	NCIMB 8653	R	+	+	—	+	—	—	—	6
	<i>C. acetobutylicum</i>	NRRL B591	R	+	+	—	+	—	—	—	6
	<i>C. acetobutylicum</i>	NRRL B597	R	+	+	—	+	—	—	—	ND
	“ <i>C. madisonii</i> ”	214	R	+	+	—	—(L)	—	—	—	6
“ <i>C. madisonii</i> ”	4J9	R	+	+	—	—(L)	—	—	—	6	
7	<i>C. acetobutylicum</i>	ATCC 39058	R	+	+	—	—	—	—	—	7
8	<i>C. beijerinckii</i>	NRRL B593	R	+	+	—	—	—	—	—	8
9	<i>C. beijerinckii</i>	NRRL B592	R	—	+	—	—	—	—	—	9
	<i>C. beijerinckii</i>	NRRL B466	R	—	+	—	—	—	—	—	9
	<i>C. acetobutylicum</i>	NRRL B596	R	—	+	—	—	—	—	—	9

^a S, susceptible to 10 and 100 ng of rifampin per disc; R, resistant to 10 and 100 ng of rifampin per disc.

^b -, no inhibitory effect; +, inhibitory effect.

^c - , no cell lysis; + , cell lysis; L, lysogen.

^d ND, not determined. a and b indicate variations of the same DNA fingerprint.

^c The partial region of the 16S rRNA gene amplified by PCR primers PCRA and PCRB was sequenced.

FIG. 1. Schematic diagram showing the locations of the amplification and sequencing primers on the 16S rRNA gene. The shaded area represents the partial 16S rRNA gene sequence region examined (positions 830 to 1383; *E. coli* numbering). —>, amplification and sequencing primer; <—, sequencing primer. Primer designations are indicated below the primers. The size and position of each overlapping PCR product are indicated. The box represents the 1,541-bp 16S rRNA gene.

1 min and a final cycle consisting of incubation at 92°C for 1 min, at 55°C for 1 min, and at 72°C for 5 min. An aliquot (5 µl) of each PCR mixture was analyzed by agarose gel electrophoresis to confirm that products of the correct size were amplified. The PCR products were purified by using a QIAquick-spin PCR purification kit (QIAGEN) according to the instructions of the manufacturer.

DNA sequencing. Sequencing reactions were carried out by using a PRISM ready reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) according to the instructions of the manufacturer. Sequence reaction mixtures were electrophoresed by using a model ABI 373A automated DNA sequencer (Applied Biosystems).

Sequence analyses. The 16S rRNA sequences were aligned by using the LaserGene application software MegAlign (DNASTAR, Inc.) for Apple Macintosh computers, which employed the algorithm CLUSTAL V. The partial 16S rRNA sequences obtained were aligned with a set of partial *Clostridium* sequences, the full sequences of which were retrieved from the GenBank database. In a subsequent alignment the complete 16S rRNA gene sequences of *C. acetobutylicum* ATCC 824^T, NCP 262, and NCIMB 8052^T and "*C. saccharoperbutylacetonicum*" N1-4 were analyzed by comparing them with the reference sequences. Phylogenetic dendrograms were produced by using the neighbour-joining method of Saitou and Nei (40).

Nucleotide sequence accession numbers. The partial 16S rRNA gene sequences determined in this study (positions 830 to 1383; *E. coli* numbering) are available from the GenBank database under the following accession numbers: U16164 (*C. acetobutylicum* ATCC 39058), U16167 (*C. beijerinckii* NRRL B592), U16168 (*C. beijerinckii* NRRL B593), U16169 ("*C. saccharoperbutylacetonicum*" N1-504), U16170 (*C. acetobutylicum* NCP 193), U17030 (*C. acetobutylicum* DSM 792^T), and U17099 (*C. acetobutylicum* NCIMB 8653). The complete 16S rRNA gene sequences which we determined are available from the GenBank database under accession numbers U16122 ("*C. saccharoperbutylacetonicum*" N1-4), U16147 (*C. acetobutylicum* NCP 262), U16165 (*C. acetobutylicum* NCIMB 8052^T), and U16166 (*C. acetobutylicum* ATCC 824^T).

RESULTS

Biotyping procedures. Each of the 55 solvent-producing strains included in this survey was subjected to biotyping analyses, including determination of susceptibility to rifampin, bacteriocin typing, and phage typing. On the basis of the results which we obtained the strains could be divided into several biotypes (Table 2).

It has been observed previously that different industrial strains of solvent-producing clostridia vary widely in their susceptibility to rifampin. All of the strains used in this study were either susceptible to <200 ng of rifampin per ml or resistant to >2,000 ng of rifampin per ml. The strains belonging to groups 1 and 2 were susceptible to the 10- and 100-ng rifampin discs (Table 2). However, the group 1 strain preparations exhibited zones of inhibition surrounding the 100-ng discs which ranged from 2.5 to 4 mm wide, whereas with the group 2 strain preparations the zones of growth inhibition ranged from 4 to 7 mm wide. The strains belonging to the other seven groups were all resistant to the 100-ng rifampin discs.

A mitomycin C-induced bacteriocin produced by *C. aceto-*

butylicum NCP 262 and a bacteriocin produced constitutively by *C. acetobutylicum* NCP 258 were found to inhibit the growth of a number of solvent-producing clostridial strains (Table 2). Neither of these bacteriocin-like agents inhibited the growth of the clostridial strains belonging to groups 1, 2, and 5; the growth of strains belonging to groups 3, 6, 7, and 8 was inhibited in the presence of both bacteriocins; and the growth of strains belonging to group 4, as well as the three group 9 strains, was inhibited by the bacteriocin-like agent from *C. acetobutylicum* NCP 262, but these strains were resistant to the inhibitory agent isolated from NCP 258.

Phages CA1, CA2, HMT, and HM2 belonging to the *Podoviridae*, phage HM3 belonging to the *Myoviridae*, and phages CMX and HM7 belonging to the *Siphoviridae* were used in typing experiments. Bacteriophages CA1 and CA2 infected only the rifampin-resistant NCP industrial strains belonging to group 5. Bacteriophage CMX, which was isolated from "*C. madisonii*" 4J9 by using mitomycin C induction, infected all of the culture collection strains included in group 6. However, "*C. madisonii*" 214 and 4J9, two strains which belong to group 6, were resistant to infection by phage CMX. Both of these strains were found to be lysogenic (unpublished data). Phages HMT, HM2, HM3, and HM7 infected only strains labeled "*C. saccharoperbutylacetonicum*." Temperate phage HMT infected the indicator strain "*C. saccharoperbutylacetonicum*" N1-4 (= ATCC 13564) but not lysogenic strain ATCC 27021. Two morphologically different bacteriophages, HM2 and HM3, infected group 3 strains N1-4 and ATCC 13564, and phage HM7 infected only group 4 strains N1-504 and ATCC 27022. Strains belonging to groups 1, 2, 7, 8, and 9 were resistant to all of the phages included in this study (Table 2).

DNA fingerprint analysis. Several restriction endonucleases were tested in this study, and we found that the 8-base recognition enzymes either did not cleave the genomes of *C. acetobutylicum* NCP 262 and NCP 193 or did not produce enough fragments for strain comparisons. The restriction endonucleases containing the rare CTAG tetranucleotide in their recognition sites produced too many fragments, as did many of the G+C-rich 6-base enzymes. We found that *ApaI*, *BssHII*, *EagI*, and *SmaI* were the most useful enzymes for strain comparisons as they generated appropriate numbers of fragments in the 9- to 1,600-kb range for DNA fingerprint comparisons. These enzymes were used routinely for DNA fingerprint analyses of all of the clostridial strains.

Separation of the DNA fragments by PFGE revealed that the 55 solvent-producing clostridial strains examined fell into nine clearly distinguishable DNA fingerprint groups (Table 2);

FIG. 2. DNA fingerprints of solvent-producing clostridial strains belonging to the nine groups. (A) *Bss*HII restriction endonuclease digests of genomic DNAs from group 1 strains. Program 2 electrophoresis conditions. (B) *Apa*I restriction endonuclease digests of genomic DNAs from group 2 strains. Program 2 electrophoresis conditions. (C) *Eag*I restriction endonuclease digests of genomic DNAs from group 5 strains. Program 1 electrophoresis conditions. (D) *Sma*I restriction endonuclease digests of genomic DNAs from group 6 strains. Program 1 electrophoresis conditions. (E) *Sma*I restriction endonuclease digests of genomic DNAs from group 3, 4, 7, 8, and 9 strains. Program 1 electrophoresis conditions. Strain designations are indicated. Lanes λ + λ /HindIII and λ ladder contained the Low Range PFG Markers (New England Biolabs) and Lambda Ladder PFG Markers (New England Biolabs), respectively. All sizes are in kilobases.

the strains in each group exhibited similar or identical DNA profiles (Fig. 2), no matter which of the restriction endonucleases were used.

The strains belonging to group 1 included strains ATCC

824^T, DSM 792^T, and NRRL B527^T, but not *C. acetobutylicum* NCIMB 8052^T. These strains were found to have similar DNA profiles (Fig. 2A and Table 2). We observed some differences in the *Bss*HII DNA fingerprints of strains DSM 1731 (= ATCC

FIG. 3. DNA fingerprints of the prototype strains of the nine groups. (A) *Bss*HII restriction endonuclease digests of genomic DNA. Program 2 electrophoresis conditions. (B) *Eag*I restriction endonuclease digests of genomic DNA. Program 1 electrophoresis conditions. Strain designations are indicated. Lanes $\lambda + \lambda$ /HindIII and λ ladder contained the Low Range PFG Markers (New England Biolabs) and Lambda Ladder PFG Markers (New England Biolabs), respectively. All sizes are in kilobases.

4259 = NCIMB 619 = NRRL B530) and ATCC 824^T (Fig. 2A). As noted previously by Wilkinson and Young (46), the *Apa*I and *Sma*I fragment patterns of ATCC 824^T were very similar to the corresponding *Apa*I and *Sma*I profiles of DSM 1731 (data not shown). Some fragments of *C. acetobutylicum* ATCC 39236, an asporogenic mutant of ATCC 4259 (24), resembled fragments in the *Bss*HII digest of DSM 1731, and the remainder of the ATCC 39236 *Bss*HII fragments comigrated with the ATCC 824^T fragments (Fig. 2A). The fingerprints of the group 1 strains from which nondegraded DNAs were extracted were identical to the DNA fingerprint of ATCC 824^T (Fig. 2A and Table 2). Extraction of intact DNA from several of the group 1 strains was not always successful. Wilkinson and Young reported that a key modification which was necessary for optimal DNA extraction from strain NCIMB 8052^T was inclusion of hypertonic sucrose in the lysis buffer (46). However, this modification did not overcome the problem of DNA degradation encountered with the group 1 strains. Instead, a modification of the standard procedure which involved incubating the cell suspension with protease prior to lysozyme treatment was used. Genomic DNA was extracted successfully from DSM 1737 and DSM 1733 by using this procedure.

Some variation in the DNA fingerprints was also observed with the group 2 strains (Fig. 2B and Table 2), which included the rifampin-susceptible NCP industrial strains and culture

collection strain NRRL B643. Strains NCP 258 and NRRL B643 produced indistinguishable DNA fingerprints, and these fingerprints were similar but not identical to the fingerprints of the other NCP strains belonging to group 2. Groups 3 and 4 both contained saccharolytic butanol-producing strains of "*C. saccharoperbutylacetonicum*." The DNA fragment patterns of "*C. saccharoperbutylacetonicum*" N1-4 and N1-504 had only a small number of comigrating fragments (Fig. 2E). The group 5 strains, which included the bacteriophage CA1- and CA2-susceptible NCP strains, all produced identical DNA profiles (Fig. 2C), as did all of the group 6 strains which were sensitive to bacteriophage CMX (Fig. 2D). The biotypes exhibited by *C. acetobutylicum* ATCC 39058 (group 7) and *C. beijerinckii* NRRL B593 (group 8) were indistinguishable (Table 2); however, on the basis of their different DNA fingerprints (Fig. 2E), these two strains were assigned to separate groups. Group 9 contained two *C. beijerinckii* strains (NRRL B592 and NRRL B466), as well as a *C. acetobutylicum* strain (NRRL B596), all of which produced identical fingerprint profiles (Fig. 2E). High levels of heterogeneity in restriction fragment patterns were observed in the nine groups of solvent-producing clostridia; examples of these patterns are shown in Fig. 3.

DNA sequence analysis of the partial 16S rRNA gene. Nucleotide sequencing of the 16S rRNA gene from representative strains belonging to the nine groups was performed to determine the taxonomic and phylogenetic relationships of these

FIG. 4. Unrooted phylogenetic dendrogram showing the taxonomic positions of the solvent-producing strains *C. acetobutylicum* ATCC 824^T, DSM 792^T, NCP 193, NCIMB 8052^T, NCIMB 8653, and ATCC 39058, *C. beijerinckii* NRRL B592 and NRRL B593, and "*C. saccharoperbutylacetonicum*" N1-4 and N1-504, based on the partial 16S rRNA gene sequences examined (positions 830 to 1383; *E. coli* numbering). The scale bar indicates evolutionary distances.

organisms. The high levels of similarity among the DNA fingerprint patterns of all of the strains belonging to each group meant that one strain from each group could be selected as the group prototype strain. In each case the prototype strain selected was the strain which had been worked with most extensively, had been cited most frequently in the literature, or was the only strain belonging to the group. The following strains were used in this analysis: type strain ATCC 824 for group 1; *C. acetobutylicum* NCP 262 for group 2; "*C. saccharoperbutylacetonicum*" N1-4 and N1-504 for groups 3 and 4, respectively; *C. acetobutylicum* NCP 193, NCIMB 8052^T, and ATCC 39058 for groups 5, 6, and 7, respectively; and *C. beijerinckii* NRRL B593 and NRRL B592 for groups 8 and 9, respectively.

The partial 16S rRNA gene sequences (positions 830 to 1383; *E. coli* numbering) of the nine prototype strains were determined and compared with the previously described sequences of other clostridia. In addition, the partial 16S rRNA gene sequences of strains DSM 792^T and NCIMB 8653 were also determined. Levels of sequence similarity were calculated from the results of a comparison of 550 nucleotides, and a phylogenetic tree (Fig. 4) was constructed from a matrix of derived evolutionary distance values. The values obtained for the solvent-producing clostridial strains and some of the *Clostridium* species with which the solvent-producing strains exhib-

ited high levels of homology are shown in Table 3. Our results demonstrated that prototype strains NCP 193 (group 5), NCIMB 8052^T and NCIMB 8653 (group 6), ATCC 39058 (group 7), NRRL B593 (group 8), and NRRL B592 (group 9) were 100% identical to one another as well as to *C. beijerinckii* DSM 791^T. "*C. saccharoperbutylacetonicum*" N1-4 (group 3) and "*C. saccharoperbutylacetonicum*" N1-504 (group 4) also had identical 550-nucleotide partial 16S rRNA gene sequences. The partial sequences of type strains *C. acetobutylicum* ATCC 824 and DSM 792 were identical to each other but exhibited only 88.2% similarity with the 16S rRNA gene sequence of the equivalent organism *C. acetobutylicum* NCIMB 8052^T.

The findings described above indicate that the nine groups established by biotyping and DNA fingerprinting analyses could be placed into four major taxonomic groups on the basis of the levels of relatedness of their partial 16S rRNA gene sequences. Taxonomic group I contained the strains belonging to biotype and DNA fingerprint group 1, which was represented by the amylolytic organism *C. acetobutylicum* ATCC 824^T. Taxonomic groups II, III, and IV contained the remaining eight biotype and DNA fingerprint groups and were represented by the saccharolytic strains *C. acetobutylicum* NCP

TABLE 3. Levels of sequence similarity and evolutionary distances based on an alignment of 550 nucleotides of the partial 16S rRNA gene sequences (positions 830 to 1383; *E. coli* numbering) of 11 solvent-producing clostridial strains and other clostridia

Species or strain	% Sequence similarity or evolutionary distance ^a																
	<i>C. beijerinckii</i> NRRL B592	<i>C. beijerinckii</i> NRRL B593	<i>C. beijerinckii</i> DSM 791 ^T	<i>C. acetobutylicum</i> NCP 193	<i>C. acetobutylicum</i> ATCC 39058	<i>C. acetobutylicum</i> NCIMB 8052 ^T	<i>C. acetobutylicum</i> NCIMB 8653	<i>C. beijerinckii</i> NCIMB 9362 ^T	<i>C. acetobutylicum</i> NCP 262	<i>C. puniceum</i>	" <i>C. saccharoperbutylacetonicum</i> " N1-4	" <i>C. saccharoperbutylacetonicum</i> " N1-504	<i>C. chartatabidum</i>	<i>C. acetobutylicum</i> ATCC 824 ^T	<i>C. acetobutylicum</i> DSM 792 ^T	<i>C. sardiniense</i>	<i>C. collagenovorans</i>
<i>C. beijerinckii</i> NRRL B592		100.0	100.0	100.0	100.0	100.0	100.0	99.6	97.6	94.7	98.0	98.0	96.9	88.2	88.2	87.3	88.5
<i>C. beijerinckii</i> NRRL B593	0.0		100.0	100.0	100.0	100.0	100.0	99.6	97.6	94.7	98.0	98.0	96.9	88.2	88.2	87.3	88.5
<i>C. beijerinckii</i> DSM 791 ^T	0.0	0.0		100.0	100.0	100.0	100.0	99.6	97.6	94.7	98.0	98.0	96.9	88.2	88.2	87.3	88.5
<i>C. acetobutylicum</i> NCP 193	0.0	0.0	0.0		100.0	100.0	100.0	99.6	97.6	94.7	98.0	98.0	96.9	88.2	88.2	87.3	88.5
<i>C. acetobutylicum</i> ATCC 39058	0.0	0.0	0.0	0.0		100.0	100.0	99.6	97.6	94.7	98.0	98.0	96.9	88.2	88.2	87.3	88.5
<i>C. acetobutylicum</i> NCIMB 8052 ^T	0.0	0.0	0.0	0.0	0.0		100.0	99.6	97.6	94.7	98.0	98.0	96.9	88.2	88.2	87.3	88.5
<i>C. acetobutylicum</i> NCIMB 8653	0.0	0.0	0.0	0.0	0.0	0.0		99.6	97.6	94.7	98.0	98.0	96.9	88.2	88.2	87.3	88.5
<i>C. beijerinckii</i> NCIMB 9362 ^T	0.4	0.4	0.4	0.4	0.4	0.4	0.4		97.3	94.4	97.6	97.6	96.5	88.4	88.4	87.5	88.7
<i>C. acetobutylicum</i> NCP 262	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.6		96.6	99.5	99.5	95.3	88.9	88.9	88.0	89.6
<i>C. puniceum</i>	1.7	1.7	1.7	1.7	1.7	1.7	1.7	2.0	0.9		96.7	96.7	94.4	89.2	89.2	87.5	88.1
" <i>C. saccharoperbutylacetonicum</i> " N1-4	0.9	0.9	0.9	0.9	0.9	0.9	0.9	1.3	0.5	0.7		100.0	95.6	89.5	89.5	88.7	90.1
" <i>C. saccharoperbutylacetonicum</i> " N1-504	0.9	0.9	0.9	0.9	0.9	0.9	0.9	1.3	0.5	0.7	0.0		95.6	89.5	89.5	88.7	90.1
<i>C. chartatabidum</i>	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.6	1.8	2.6	1.5	1.5		90.0	90.0	88.6	89.2
<i>C. acetobutylicum</i> ATCC 824 ^T	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.1	7.6	7.3	7.1	7.1	7.3		100.0	97.1	94.1
<i>C. acetobutylicum</i> DSM 792 ^T	7.3	7.3	7.3	7.3	7.3	7.3	7.3	7.1	7.6	7.3	7.1	7.1	7.3	0.0		97.1	94.1
<i>C. sardiniense</i>	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.6	7.1	7.2	6.6	6.6	6.2	0.7	0.7		92.3
<i>C. collagenovorans</i>	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.0	7.2	7.2	6.6	6.6	6.8	2.2	2.2	1.5	

^a The values on the upper right are levels of sequence similarity, and the values on the lower left are evolutionary distances.

262, "*C. saccharoperbutylacetonicum*" N1-4, and *C. acetobutylicum* NCIMB 8052^T, respectively.

Intact chromosomal DNA could not be obtained from a number of group 1 strains (Table 2). Hence, DNA fingerprints, the main criterion for separating the group 1 strains from the group 2 strains, could not be determined for strains ATCC 8529 (= DSM 1738), DSM 1732 (= NCIMB 2951), NCIMB 6443, and ATCC 43084. To confirm that these strains belonged in group 1, the partial 16S rRNA gene regions from partially degraded DNAs of ATCC 8529, DSM 1732, NCIMB 6443, and ATCC 43084 embedded in agarose plugs were amplified and sequenced. All four strains had a 550-bp sequence identical to the sequence obtained for the equivalent regions of ATCC 824^T and DSM 792^T, confirming that they belong to group 1 (Table 2).

DNA sequence analysis of the complete 16S rRNA gene. To establish more reliable phylogenetic positions for the four taxonomic groups of solvent-producing clostridia, the complete 16S rRNA gene of the representative strain of each taxonomic group was amplified. Almost complete 16S rRNA gene sequences were determined for *C. acetobutylicum* ATCC 824^T (1,411 bases), *C. acetobutylicum* NCP 262 (1,416 bases), *C. acetobutylicum* NCIMB 8052^T (1,416 bases), and "*C. saccharoperbutylacetonicum*" N1-4 (1,416 bases). These sequences were aligned with other *Clostridium* 16S rRNA gene sequences, levels of sequence similarity and evolutionary distances were calculated (based on 1,334 nucleotides, ranging from position 100 to position 1434 [*E. coli* numbering]), and a phylogenetic dendrogram was constructed (Fig. 5). This was done in accordance with previously published alignments of

Clostridium species, in which the 5' ends of the 16S rRNA gene sequences were omitted to avoid possible misalignment of variable region V1 of clostridial 16S rRNA gene sequences (16, 23). The levels of sequence similarity and evolutionary distances for the closest relatives of the four solvent-producing clostridial strains are shown in Table 4. Our results showed that type strain *C. acetobutylicum* ATCC 824 was phylogenetically distant from saccharolytic strains *C. acetobutylicum* NCIMB 8052^T, "*C. saccharoperbutylacetonicum*" N1-4, and *C. acetobutylicum* NCP 262 (evolutionary distances, 7.4 to 7.7%) (Table 4). NCP 262 was found to be more closely related to, yet distinct from N1-4 (level of sequence similarity, 98.9%) and NCIMB 8052^T (level of sequence similarity, 98.2%). "*C. saccharoperbutylacetonicum*" N1-4 was phylogenetically closely related to but also distinct from NCIMB 8052^T (evolutionary distance, 0.7%). Our results confirmed that *C. acetobutylicum* NCIMB 8052^T exhibited 100% sequence homology with type strain *C. beijerinckii* DSM 791 (Table 4).

DISCUSSION

Biotyping and DNA fingerprint analysis. Although bacteriocin typing and bacteriophage typing have been used extensively for identifying some pathogenic *Clostridium* species, they have not been used previously to differentiate solvent-producing clostridia. A combination of bacteriocin typing, bacteriophage typing, and rifampin susceptibility testing provided a simple way to group the various strains of solvent-producing clostridia. These biotyping procedures were readily carried out, reproducible, and easy to interpret.

FIG. 5. Unrooted phylogenetic dendrogram showing the taxonomic positions of *C. acetobutylicum* ATCC 824^T, NCP 262, and NCIMB 8052^T and "*C. saccharoperbutylacetonicum*" N1-4, based on the complete 16S rRNA gene sequences (positions 100 to 1434; *E. coli* numbering). The scale bar indicates evolutionary distances.

The bacteriophages of both solvent-producing and pathogenic clostridia have been reported to exhibit narrow host ranges (34). Hongo and Murata investigated the host ranges of 12 HM phages which were isolated from abnormal industrial fermentations (12). These phages were tested with 31 different saccharolytic and amylolytic strains of butanol-producing, butyric acid-producing, and isopropanol-producing bacteria belonging to several different *Clostridium* species, and they reportedly infected only strains of "*C. saccharoperbutylacetonicum*" (12). Similar results were obtained in this study. Phages CA1, CA2, and CMX also exhibited narrow host ranges, and phage susceptibility was confined to strains belonging to the same DNA fingerprint group.

The use of PFGE for genomic DNA analysis has proved to be useful for differentiating strains of closely related bacteria. A DNA fingerprint analysis of the solvent-producing clostridial strains in which PFGE was used was a highly discriminatory method for comparing strains which had been differentiated by biotyping.

On the basis of the results obtained in biotyping and DNA fingerprint analyses it was possible to divide the 55 strains of solvent-producing clostridia which we examined into nine groups. The genomic DNA restriction fragment profiles obtained for strains belonging to the same group exhibited high levels of similarity, whereas the DNA profiles obtained for members of different groups were markedly different. The biotyping and DNA fingerprint analysis results exhibited a high level of correlation, indicating that the strains belonging to

each group were phenotypically and genotypically closely related. However, these results did not provide any insight into the phylogenetic relationships among the nine groups.

Attempts to extract intact chromosomal DNAs from a number of the amylolytic strains belonging to group 1 were unsuccessful, which meant that it was not possible to obtain restriction endonuclease profiles for these strains. Similar problems with DNA degradation have been encountered by other researchers during preparation of DNA from *Clostridium difficile*, *Clostridium perfringens*, and *C. acetobutylicum* for PFGE analysis (22, 46). The mechanism of degradation is not known, but degradation is presumed to be due to nuclease activity. Placement of these strains in group 1 was based on their 550-bp 16S rRNA gene sequences.

DNA sequence analysis of the partial 16S rRNA gene. A DNA sequence analysis of the partial 16S rRNA genes from one or more representative strains belonging to each of the nine groups was performed to determine the phylogenetic relationships among the groups. An alignment of 16S rRNA gene sequences obtained from GenBank showed that the region corresponding to positions 830 to 1383 (*E. coli* numbering) consisted of a relatively variable region of the 16S rRNA gene. This segment of the 16S rRNA gene provided a quick and reliable method for comparing the prototype strains of solvent-producing clostridia belonging to each group. On the basis of the partial 16S rRNA gene sequences the nine biotype and DNA fingerprint groups could be assigned to four taxonomic groups.

Taxonomic group I. Taxonomic group I contained the amylolytic *C. acetobutylicum* strains belonging to biotype and DNA fingerprint group 1. This group included *C. acetobutylicum* type strain ATCC 824 (= DSM 792 = NRRL B527) as described by Weyer and Rettger (45), as well as the *C. acetobutylicum* Weizmann industrial strain and various other strains which were isolated during the early part of this century for their ability to produce solvents from starch-based substrates, such as maize. *C. acetobutylicum* ATCC 4259 (= DSM 1731 = NCIMB 619 = NRRL B530), which was originally deposited by Thaysen in 1920, appears to be a derivative of the original patented Weizmann strain. In addition, strains DSM 1732 and NCIMB 2951, which were originally deposited by Thaysen, were identified as derivatives of the original Weizmann strain. A strain deposited by Hall in the ATCC and listed as ATCC 3625 (= DSM 1737 = NRRL B529) was derived from an industrial butyl culture and therefore could represent another variant of the original Weizmann culture (27). Strain ATCC 862 was independently isolated by Donker and was deposited by Kluyver in the ATCC in 1926. McCoy and McClung reported that this strain resembled the other amylolytic strains of *C. acetobutylicum* included in their study (27). This strain is no longer held by the ATCC, and, unfortunately, attempts to propagate the equivalent organism strain NRRL B528 were unsuccessful. An asporogenic mutant derived from *C. acetobutylicum* ATCC 4259 and listed as ATCC 39236 also fell into this taxonomic group, as did the butanol-producing organism *C. acetobutylicum* ATCC 43084, which was isolated from cassava roots, a starch-based substrate.

Taxonomic group II. Taxonomic group II contained saccharolytic *C. acetobutylicum* strains belonging to biotype and DNA fingerprint group 2. This group included *C. acetobutylicum* NCP 262 and several other closely related strains utilized by NCP in South Africa from 1945 to 1983 for industrial production of acetone and butanol. In addition, this taxonomic group contained strain NRRL B643, which was deposited in the NRRL culture collection by CSC in 1946 as an example of one of their main production strains. This strain appears to be the

TABLE 4. Levels of sequence similarity and evolutionary distances based on an alignment of approximately 1,320 nucleotides of the 16S rRNAs (positions 100 to 1434; *E. coli* numbering) of four solvent-producing clostridial strains and other clostridia

Species or strain	% Sequence similarity or evolutionary distance ^a								
	<i>C. acetobutylicum</i> ATCC 8052 ^T	<i>C. beijerinckii</i> DSM 791 ^T	<i>C. beijerinckii</i> NCIMB 9362 ^T	" <i>C. saccharoperbutylacetonicum</i> " N1-4	<i>C. acetobutylicum</i> NCP 262	<i>C. puniceum</i>	<i>C. acetobutylicum</i> ATCC 824 ^T	<i>C. collagenovorans</i>	<i>C. sardiniense</i>
<i>C. acetobutylicum</i> ATCC 8052 ^T		100.0	99.7	98.9	98.2	97.2	90.0	89.9	91.5
<i>C. beijerinckii</i> DSM 791 ^T	0.0		99.7	98.9	98.2	97.2	90.0	89.9	91.5
<i>C. beijerinckii</i> NCIMB 9362 ^T	0.3	0.3		98.7	98.1	96.9	90.0	89.9	91.5
" <i>C. saccharoperbutylacetonicum</i> " N1-4	0.7	0.7	0.8		98.9	98.1	90.5	90.7	92.1
<i>C. acetobutylicum</i> NCP 262	1.3	1.3	1.4	1.0		97.6	90.4	90.6	91.8
<i>C. puniceum</i>	1.3	1.3	1.5	0.9	1.3		90.5	89.6	91.8
<i>C. acetobutylicum</i> ATCC 824 ^T	7.7	7.7	7.6	7.4	7.7	7.1		92.2	93.4
<i>C. collagenovorans</i>	8.2	8.2	8.1	7.8	8.0	8.0	5.9		91.7
<i>C. sardiniense</i>	5.8	5.8	5.8	5.8	5.7	5.3	4.8	5.1	

^a The values on the upper right are levels of sequence similarity, and the values on the lower left are evolutionary distances.

only culture of an organism originally designated "*Clostridium saccharo-butyl-aceticum-liquefaciens*" to be deposited in a culture collection. The initial patent describing this new saccharolytic member of the genus *Clostridium* was filed by Arzberger in 1938 (2). This organism was utilized by CSC as its main production strain in the United States, as well as by the sister Commercial Solvents plant at Bromborough, Great Britain, for the majority of their AB fermentations with molasses following its introduction in 1938. NCP began performing the AB fermentation process in South Africa in 1936 by using maize mash as the fermentation substrate (42). In 1945 the NCP plant was converted from maize to blackstrap molasses by using the "*C. saccharo-butyl-aceticum-liquefaciens*" strains supplied by CSC in the United States and by Commercial Solvents in England (32). All of the NCP strains belonging to taxonomic group II appear to have been derived from the original industrial, saccharolytic, solvent-producing strain.

Taxonomic group III. Taxonomic group III consisted of the "*C. saccharoperbutylacetonicum*" strains belonging to biotype and DNA fingerprint groups 3 and 4. The original "*C. saccharoperbutylacetonicum*" strain, strain N1-4 (= ATCC 13564), was isolated from soil in Japan by Hongo and Nagata in 1959 and was patented by Hongo in 1960 (11, 12). This strain was used by the Sanraku Distiller's Company for industrial production of acetone and butanol from molasses. However, the fermentation process with this strain was beset by phage infections, with contamination occurring 12 times in 1 year (12). Strain N1-504 (= ATCC 27022), which reportedly was a phage-resistant mutant of the original strain N1-4, exhibited an entirely different spectrum of phage susceptibility than all of the other phage-resistant mutant strains derived from N1-4 (34). The differences in the biotype characteristics and DNA fingerprints of N1-4 and N1-504 suggest that the latter strain arose as a separate isolate rather than a phage-resistant mutant of strain N1-4. However, the partial 16S rRNA gene sequences of N1-4 and N1-504 were identical, suggesting that N1-504 is a derivative of parent strain N1-4.

Taxonomic group IV. Taxonomic group IV was the largest of the four taxonomic groups and contained the saccharolytic strains belonging to biotype and DNA fingerprint groups 5 through 9. All of the strains belonging to biotype and DNA fingerprint group 5 originated from the NCP strain collection, and no examples of this group appear to have been deposited in any of the major culture collections. These strains were used by NCP in South Africa in combination with the NCP strains belonging to group 2 for industrial production of solvents from 1945 until 1983. Although the group 5 NCP strains almost certainly originated from solvent-producing clostridial strains supplied by CSC, their origins and development remain obscure.

The strains belonging to biotype and DNA fingerprint group 6 include type strain *C. acetobutylicum* NCIMB 8052, which was originally deposited in the ATCC. This strain has been worked with extensively by researchers in Great Britain. It is apparent from the biotyping and DNA fingerprint analysis results that this strain does not resemble the *C. acetobutylicum* type strains deposited in the ATCC, DSM, and NRRL and from the 16S rRNA gene sequence analysis results that it is identical to the *C. beijerinckii* type strain. The director of the NCIMB has been informed of this anomaly, and users are being notified accordingly. The original strains of solvent-producing clostridia belonging to group 6 appear to have been isolated and developed initially by CSC as industrial production strains as part of the program to convert the AB fermentation process from a starch-based substrate to a sugar-based substrate. Strains patented under the name "*Clostridium saccharo-acetobutylicum*" appear to have been the first saccharolytic cultures isolated by CSC which produced high yields of solvents from molasses. The initial patent for this strain was obtained by Arzberger in 1936 (1). This patent was followed by two additional patents which described variants of "*C. saccharo-acetobutylicum*," one obtained by Woodruff et al. in 1937 (48) and one obtained by McCoy in 1938 (28). This bacterium appears to have been used as a production strain for fermen-

tation of molasses both by CSC in the United States and at the newly opened Commercial Solvents fermentation plant in the United Kingdom from around 1935 until it was superseded by "*C. saccharo-butyl-aceticum-liquefaciens*." A culture of a prototype strain of "*C. saccharo-acetobutylicum*" used by CSC was deposited in the NRRL culture collection by McCoy in 1945 and is currently listed as *C. acetobutylicum* NRRL B591. The DNA fingerprint of this strain was identical to the DNA fingerprints of two strains of "*C. madisonii*," a saccharolytic *Clostridium* species described and patented in 1946 by McCoy (29). "*C. madisonii*," which was ceded to the Wisconsin Alumni Research Foundation, was later used as an industrial production strain in Puerto Rico (34). The DNA fingerprint patterns of the strains described above were identical to the DNA fingerprint patterns of the other *C. acetobutylicum* ATCC, DSM, NCIMB, and NRRL strains belonging to group 6, which originally came from the Wisconsin strain collection and are listed as NCIMB 8049 (= DSM 1739 = ATCC 10132 = NRRL B594), NCIMB 6444, NCIMB 6445, and NRRL B597.

Only one strain, *C. acetobutylicum* ATCC 39058, belonged to biotype and DNA fingerprint group 7. This strain is a butanol-resistant mutant that was developed from the original parent strain (ATCC 39057) isolated by enrichment culture on Jerusalem artichoke medium at the Institut Francaise du Pétrole as part of an initiative to produce butanol and isopropanol for use as blending agents to enable methanol to be added to petrol as a fuel extender (24).

Strain NRRL B593, which was classified as a *C. beijerinckii* strain, belonged to group 8. Group 9 contained three strains, strain NRRL B596 (listed as a *C. acetobutylicum* strain) and strains NRRL B592 and NRRL B466 (classified as *C. beijerinckii* strains). No solvent-producing strains classified as *C. beijerinckii* strains were used as industrial production strains; however, a number of strains which were patented as "*Clostridium propyl-butylicum*" (31), "*Clostridium viscifaciens*" (41), and "*Clostridium amylo-saccharobutyl-propylicum*" (3) strains were similar in some respects to strains classified as *C. beijerinckii* strains (19).

Phylogenetic relationships and nomenclature. On the basis of their partial 16S rRNA gene sequences, the prototype strains of solvent-producing clostridia were found to fall in rRNA homology group I of Johnson and Francis (18), which recently has been referred to by Collins et al. as cluster I (6). To determine the genealogical relationships of the solvent-producing clostridia in homology group I or cluster I more accurately, the complete 16S rRNA gene sequences of representative prototype strains of four taxonomic groups were determined.

The results obtained from a comparative sequence analysis of complete (or almost complete) 16S rRNA sequences confirmed that the type strain of *C. acetobutylicum*, ATCC 824, which was described by Weyer and Rettger in 1927 (45), is only distantly related to the saccharolytic strains of solvent-producing clostridia. This result is consistent with the findings of Johnson and Francis (18) which placed ATCC 824^T in subgroup IJ of rRNA homology group I, which was quite distinct from subgroup IA containing *C. beijerinckii*, *C. butyricum*, and the saccharolytic, nonproteolytic strains of *C. botulinum*. The strains which form taxonomic group I include the original strains classified as *C. acetobutylicum*, and therefore strains belonging to this group should retain the species name *C. acetobutylicum*.

Taxonomic groups II, III, and IV, represented by strains NCP 262, N1-4, and NCIMB 8052^T, respectively, exhibited high levels of 16S rRNA gene sequence similarity (>97%).

According to the criteria recommended by Stackebrandt and Goebel, it is not possible by using 16S rRNA gene sequences alone to determine whether these three taxonomic groups of solvent-producing clostridia constitute three separate species or are variants of a single species (43). At this level of similarity DNA-DNA hybridization techniques provide a superior method for discriminating between species and strains; a DNA reassociation value of 70% or more indicates that organisms belong to the same species (43). The results obtained from genomic DNA-DNA hybridization studies (17) indicate that these three taxonomic groups of saccharolytic solvent-producing clostridia do constitute three separate species, which means that the strains belonging to these groups should be reclassified.

Taxonomic group II contains industrial strains which were used by both CSC and NCP as their main production strains during the latter stages of operation of the AB fermentation process. In 1945 CSC deposited a culture of their main production strain in the NRRL culture collection, and it appears that the original name used to identify this strain in the patent literature was "*C. saccharo-butyl-aceticum-liquefaciens*." Strain NRRL B643 was found to have a genomic DNA profile identical to that of NCP 258 and very similar to that of NCP 262; both NCP 258 and NCP 262 were derived from cultures supplied to NCP by CSC in 1945 when the NCP fermentation process was converted from maize mash to molasses. The similarity of the genomic DNA fingerprints produced by all of the NCP strains belonging to this group suggests that they all descended from the original "*C. saccharo-butyl-aceticum-liquefaciens*" strain patented by CSC. As this was the original name used in the patent literature to identify strains belonging to taxonomic group II, it seems appropriate that this name should be retained despite being cumbersome.

Taxonomic group III contains strains isolated by Hongo which were described and patented as "*C. saccharoperbutylacetonicum*" strains (11). Although this taxon is not recognized as a valid species, a number of culture collections continue to list these strains under the name "*C. saccharoperbutylacetonicum*," and it seems appropriate that the original name for this group be retained.

The saccharolytic solvent-producing clostridial strains belonging to taxonomic group IV include various strains which have been named in the patent literature "*C. saccharo-acetobutylicum*" and "*C. madisonii*," as well as strains presently classified as *C. beijerinckii* and *C. acetobutylicum* strains. As determined by a comparative 16S rRNA gene sequence analysis, the representative prototype strain of taxonomic group IV is identical to the type strain of *C. beijerinckii*. Since *C. beijerinckii* is a legitimate species, it seems appropriate that all of the strains belonging to this taxonomic group be reclassified as *C. beijerinckii* strains.

From the results obtained in this study which support the original findings of Johnson and Francis (18) it is apparent that many saccharolytic strains currently classified as *C. acetobutylicum* strains differ markedly from the *C. acetobutylicum* strains originally isolated for their ability to produce solvents from starch-based substrates. We concluded that numerous saccharolytic solvent-producing clostridia which were isolated and patented from the mid-1930s onward make up an entirely different assemblage of solvent-producing clostridia and include representatives of at least three closely related but apparently distinct species.

Studies to phenotypically characterize the various groups of solvent-producing clostridia belonging to the four species are under way in our laboratory.

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ADDENDUM

After this paper was submitted, the 16S rRNA gene sequences of *C. acetobutylicum* ATCC 824^T, DSM 1731, NCIMB 8052^T, and NCP 262 were published by Wilkinson et al. (47). These authors also concluded that *C. acetobutylicum* NCIMB 8052^T exhibited 100% 16S rRNA sequence relatedness with the type strain of *C. beijerinckii* and was only distantly related to strains ATCC 824^T and DSM 1731. Wilkinson et al. also found that *C. acetobutylicum* NCP 262 is closely related to strain NCIMB 8052^T (47).

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