Cultures of "*Clostridium acetobutylicum*" from Various Collections Comprise *Clostridium acetobutylicum*, *Clostridium beijerinckii*, and Two Other Distinct Types Based on DNA-DNA Reassociation

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The best-known acetone-butanol (solvent)-producing bacterium is the Weizmann organism, *Clostridium acetobutylicum*, which was used for starch-based industrial fermentation. In the past two decades, cultures of "*C. acetobutylicum*" from various culture collections have included organisms that were isolated for sugar (molasses)-based industrial solvent production. Recent biochemical and genetic studies have revealed significant differences among some of these "*C. acetobutylicum*" strains. We used DNA-DNA reassociation to analyze 39 cultures of "*C. acetobutylicum*" and phenotypically similar organisms from major collections. The results of this study clearly identified four groups with intergroup reassociation values of less than 30%. All of the intragroup values except the value for one strain were 68% or more, which supported species status for each group. The *C. acetobutylicum* group (with ATCC 824 as the type strain) consisted of 17 cultures and had average reassociation values of 10% with the other three groups. All strains of *C. acetobutylicum* produced riboflavin in milk, and the cultures were bright yellow, which is useful for differentiating this species from the other three groups. The *Clostridium beijerinckii* group (with VPI 5481 [= ATCC 25752] as the type strain) consisted of 16 cultures and included strains NCIMB 8052 and NCP 270. Strains NCP 262 and NRRL B643 constituted the third group, whereas strain N1-4 ("*Clostridium saccharoperbutylacetonicum*") and its derivative, strain N1-4081, formed the fourth group. At present, the last two groups are each represented by only one independent strain; definitive descriptions of these two groups as two new or revived species will require further phenotypic characterization, as well as identification of additional strains. *C. beijerinckii* NCP 270, *Clostridium* sp. strain NRRL B643, and "*C. saccharoperbutylacetonicum*" were used in industrial solvent production from molasses, which confirms that the new organisms used for the sugar-based processes are distinct from *C. acetobutylicum*.

Commercial production of acetone and butanol (solvents) by fermentation began with the Weizmann process (15, 27). The best-known solvent-producing bacterium is the Weizmann organism, *Clostridium acetobutylicum*, which was used in the corn starch-based process. After the late 1930s, molasses was used as the carbon substrate in solvent production by fermentation, and newly isolated bacteria were needed to produce solvents from sugars under practical conditions. A number of the new isolates were described as distinct *Clostridium* species in patents (3, 16). The taxonomy of these organisms, however, presented a difficult problem because relatively few phenotypic traits were truly useful for differentiating these organisms from *C. acetobutylicum* or from each other (6, 23, 28). Thus, almost all historic strains of solvent-producing clostridia that are available from major culture collections have been listed as *C. acetobutylicum*.

In the past 15 years, several strains of "*C. acetobutylicum*" were subjected to detailed biochemical, physiological, and genetic analyses, and in these studies the workers detected significant differences among the strains (see reference 12 for a recent review). In several phylogenetic studies workers identified *C. acetobutylicum* (based on the 16S rRNA gene sequence of strain NCIMB 8052) as a species closely related to *Clostridium beijerinckii* (5, 9, 18), which did not agree with the previous finding based on the 23S rRNA sequences (13). On the basis of

an analysis of the products of pyrolysis of five strains (ATCC 824^T [T = type strain], DSM 1731, NCIMB 8052, NCP 262, and N1-4081) of "*C. acetobutylicum*", as well as the 16S rRNA gene sequences of strains ATCC 824^T, DSM 1731, NCIMB 8052, and NCP 262, the following three groups were identified: (i) strain NCIMB 8052; (ii) strains NCP 262 and N1-4081; and (iii) strains ATCC 824^T and DSM 1731 (29). Based on DNA relatedness values (12) and 16S rRNA gene sequences (29), it was proposed that "*C. acetobutylicum*" NCIMB 8052 is a *C. beijerinckii* strain (12, 29). By using biotyping and DNA fingerprint analysis, industrial solvent-producing clostridia were separated into nine groups (14, 16), and an analysis of a 550-bp region of the 16S rRNA gene sequences of these organisms suggested that there were four taxonomic groups (16).

These findings point out the need to examine all available "*C. acetobutylicum*" cultures to determine their correct species designations. To identify strains of a species, the DNA relatedness measurement is the most discriminative technique because it involves the entire genome (10, 26). We used DNA-DNA reassociation to analyze 39 cultures of "*C. acetobutylicum*" and phenotypically similar organisms from major collections. Our results indicated that about one-half of the available "*C. acetobutylicum*" cultures were actually *C. beijerinckii*. In this paper, we also describe two phenotypic traits that are useful for presumptive identification of *C. acetobutylicum*.

(An outline of the major findings of this study was presented at the Third International Workshop on the Regulation of Metabolism, Genetics, and Development of the Solvent-Forming Clostridia held at Northwestern University in Evanston, Ill., on 24 and 25 June 1994.)

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MATERIALS AND METHODS

Chemicals. Special chemicals were obtained from the following sources: ¹²⁵I (sodium iodide), from Dupont Biotechnology Division, Wilmington, Del.; S1 nuclease, from Pharmacia Biotech, Piscataway, N.J.; proteinase K, from AMRESCO, Solon, Ohio; salmon sperm DNA (type III), lysozyme (chicken egg white), and riboflavin, from Sigma Chemical Co., St. Louis, Mo.; and Whatman GF/F glass microfiber filters, from Fisher Scientific, Pittsburgh, Pa. Pasteurized and homogenized cow's milk was purchased from a local market.

Bacterial strains. In this study we used practically all available cultures that were labeled *C. acetobutylicum*. Several other phenotypically similar solvent-producing clostridia with different species designations were also included for comparison. Table 1 lists the sources of the strains used in this study. The dates of receipt are included for accuracy, and each species designation is the designation used by the culture collection or the investigator when the culture was received. Each culture was microscopically examined for uniformity in the shape and size of cells and spores. Strain NRRL B3179 was not included in this study because an authentic culture was not available.

The apparent relationships among the strains have been described by Johnson and Chen (12). The history of the strains from the McCoy collection was obtained from L. S. McClung (Indiana University) and references 17 and 20. The following designations were used by E. McCoy and L. S. McClung for organisms that were listed in McCoy series A and B and were included in this study: *Bacillus butylicus* A-38, A-39, A-49, and A-50, E. McCoy butyric isolates A-8, A-13, and A-14 (from soil), *C. acetobutylicum* B-3, B-4, B-10, B-14, B-15, and B-16, *C. beijerinckii* A-67, *Clostridium butylicum* A-21, and *Clostridium saccharoacetobutylicum* A-8.

Growth. Cultures were maintained in septum-stoppered screw-cap tubes or in serum bottles fitted with septum stoppers and aluminum seals (both from Bellco, Vineland, N.J.). To facilitate the initiation of growth from spores or nongrowing cells, CO₂ was added to the culture media. The media were boiled and flushed with CO₂ during cooling before they were dispensed under a N₂ atmosphere and sterilized. A potato infusion-salts-glucose medium (7) supplemented with 1 g of ammonium sulfate per liter was routinely used for preparation of inocula. Stock cultures were maintained both in the potato-based medium and in milk (1). The pasteurized and homogenized cow's milk used was either whole milk (3.8% milk fat) or low-fat milk (2% milk fat), and both types of milk were satisfactory for the study of *C. acetobutylicum*.

Spores or freeze-dried cells of the strains received were revived in the potato-based medium and incubated at 35 to 37°C. Most of the strains grew well in the potato-based medium; the only exception was strain N1-4081 (an autolysin-deficient auxotroph for proline [21]), which grew faster in media containing Trypticase and yeast extract. Solvent production was tested in Trypticase-yeast extract-glucose (TYG) medium containing 20 g of glucose per liter as described by George et al. (7), except that the cultures were grown in 3 ml of TYG medium in Bellco screw-cap anaerobic culture tubes. The cultures used for DNA isolation were grown in a medium containing (per liter) 10 g of pepticase, 5 g of yeast extract, 20 g of glucose, 1.36 g of KH₂PO₄, 0.4 g of MgSO₄, 2 g of (NH₄)₂SO₄, 1 g of asparagine, 0.5 g of cysteine-HCl, 0.01 g of biotin, 0.01 g of *p*-aminobenzoic acid, 1 ml of a mineral salts solution (7), and enough H₂O to bring the volume to 1 liter. The pH was adjusted to 6.0 before sterilization. The 400-ml cultures used for DNA isolation were grown in 1-liter Erlenmeyer flasks fitted with pressure relief valves, and each flask was flushed with a mixed gas (CO₂-H₂-N₂, 10:10:80) before inoculation. Each 400-ml culture was inoculated with 20 to 40 ml of an actively growing culture (10 to 12 h old) and incubated at 37°C with shaking for 8 to 9 h before harvesting. The cells were used immediately for DNA isolation.

Isolation of DNA. DNA was isolated by a variation of the Marmur procedure (11). The lysing solution was heated in a boiling water bath before use, and nucleic acids were dissolved in Tris-EDTA buffer before treatment with RNases and after the final step of purification. Cells of *Clostridium* sp. strain NCP 262 were converted to protoplasts (31) before lysis.

Measurement of DNA reassociation. DNAs of reference strains were labeled with ¹²⁵I by using the procedure of Johnson (11), with the following modifications: the buffer-salt solution contained 7.2 M sodium perchlorate, 0.3 mM potassium iodide, and 80 mM sodium acetate buffer (pH 4.8); the TiCl₃ solution contained 1 mg of TiCl₃ per ml in 100 mM sodium acetate buffer (pH 4.8); and phosphate buffer-mercaptoethanol was omitted from the procedure. The degree of DNA reassociation was measured by the S1 nuclease procedure (11), except that the buffer for the reaction mixture contained 13.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.0), and the reaction mixture was incubated at 60°C for 24 h. During the reassociation reaction, single-stranded, labeled DNA fragments from a reference strain were mixed with a large excess of single-stranded, unlabeled DNA fragments from a test strain or the reference strain. After duplex formation, single-stranded DNA was digested with S1 nuclease, and the duplexes were precipitated and collected on a fiberglass membrane filter. The radioactivity on the filter was measured and used to calculate similarity values.

Analysis of cellular fatty acids. The cellular fatty acid profiles were determined by the procedure described by Moore et al. (21). Each profile was compared with the profiles of the clostridia included in the VPI Anaerobic Bacteria Database, version 3.8 (MIDI, Newark, Del.).

TABLE 1. Sources, designations, and dates of receipt of strains of *C. acetobutylicum* and phenotypically similar organisms used in this study

Strain	Date of receipt (mo/yr)	Source ^a
<i>C. acetobutylicum</i> strains		
ATCC 824 ^T	11/1982	Gherna
ATCC 3625	6/1994	Gherna
ATCC 4259	11/1982	Gherna
ATCC 8529	6/1994	Gherna
ATCC 10132	11/1982	Gherna
ATCC 35702	6/1994	Gherna
ATCC 39057	6/1994	Gherna
ATCC 39236	6/1994	Gherna
ATCC 43084	6/1994	Gherna
DSM 792	12/1993	Dürre
DSM 1731	12/1993	Dürre
DSM 1732	7/1994	Dürre
NCIMB 619	11/1993	Pirrie
NCIMB 2951	11/1993	Pirrie
NCIMB 6441	11/1993	Pirrie
NCIMB 6442	11/1993	Pirrie
NCIMB 6443	11/1993	Pirrie
NCIMB 6444	11/1993	Pirrie
NCIMB 6445	11/1993	Pirrie
NCIMB 8049	11/1993	Pirrie
NCIMB 8052[57] ^b	11/1993	Pirrie
NCIMB 8052[Y] ^b	12/1993	Young
NCP 193	9/1994	Jones
NCP 262	12/1993	Woods
NCP 270	9/1994	Jones
NCP 271	9/1994	Jones
N1-4081	11/1993	Reysset
NRRL B527	2/1981	Nakamura
NRRL B528	1/1994	Nakamura
NRRL B529	1/1994	Nakamura
NRRL B591	1/1994	Nakamura
NRRL B594	1/1994	Nakamura
NRRL B643	1/1994	Nakamura
<i>C. beijerinckii</i> VPI 5481 ^T	7/1981	VPI
<i>C. butylicum</i> strains		
NRRL B466	1/1994	Nakamura
NRRL B592 ^c	2/1981	Nakamura
NRRL B593 ^c	2/1981	Nakamura
NRRL B596	1/1994	Nakamura
" <i>C. saccharoperbutylacetonicum</i> " N1-4	1/1994	Yoshino

^a Gherna, R. Gherna, American Type Culture Collection; Dürre, P. Dürre, University of Göttingen; Pirrie, R. S. Pirrie, National Collection of Industrial and Marine Bacteria; Young, M. Young, University of Wales; Jones, D. T. Jones, University of Otago; Woods, D. R. Woods, University of Cape Town; Reysset, G. Reysset, Pasteur Institute; Nakamura, L. K. Nakamura, U.S. Department of Agriculture; VPI, VPI Anaerobe Laboratory Culture Collection; Yoshino, S. Yoshino, Kyushu University.

^b NCIMB 8052[57] is a direct subculture from a May 1957 culture of NCIMB 8052. NCIMB 8052[Y] is a current laboratory culture of M. Young.

^c NRRL B592 and NRRL B593 have been identified as *C. beijerinckii* (7).

Analytical procedures. The concentrations of acetone, butanol, and isopropanol in culture supernatants were measured by gas chromatography as described previously (30).

RESULTS

Differentiation of solvent-producing clostridia. Eleven cultures were chosen as the reference strains for this study. These cultures included the type strains of *C. acetobutylicum* and *C. beijerinckii*. The other reference strains represented apparently

TABLE 2. Levels of DNA sequence similarity for *C. acetobutylicum*-like bacteria

Species	Strain	% Similarity with:										
		ATCC 824 ^T	DSM 1731	VPI 5481 ^T	NCIMB 8052[57] ^a	NCIMB 8052[Y] ^a	NRRL B594	NRRL B592	NCP 270	NCIMB 6444	NRRL B643	N1-4
<i>C. acetobutylicum</i>	ATCC 824 ^T	(100)	98	7	13	20	6	ND ^b	ND	ND	7	12
	ATCC 3625	98	102	15	13	14	16	ND	ND	ND	6	7
	ATCC 4259	99	98	12	11	10	6	ND	ND	ND	8	7
	ATCC 8529	92	104	5	10	4	5	ND	ND	ND	4	5
	ATCC 39236	101	102	17	25	22	21	ND	ND	ND	9	11
	ATCC 43084	105	110	16	20	19	18	ND	ND	ND	8	11
	DSM 792	92	96	9	8	8	6	ND	ND	ND	9	13
	DSM 1731	96	(100)	7	12	10	7	ND	ND	ND	8	6
	DSM 1732	96	99	17	17	20	24	ND	ND	ND	9	9
	NCIMB 619	96	104	5	4	6	5	ND	ND	ND	5	5
	NCIMB 2951	97	94	8	12	15	7	ND	ND	ND	8	9
	NCIMB 6441	93	99	5	5	5	5	ND	ND	ND	5	7
	NCIMB 6442	97	98	6	4	7	5	ND	ND	ND	6	14
	NCIMB 6443	97	97	6	7	6	4	ND	ND	ND	5	5
	NRRL B527	95	96	6	6	11	5	ND	ND	ND	7	5
	NRRL B528	84	87	9	10	10	9	ND	ND	ND	7	5
	NRRL B529	94	101	10	15	13	10	ND	ND	ND	7	8
<i>C. beijerinckii</i>	VPI 5481 ^T	4	10	(100)	83	73	84	76	74	57	22	18
	ATCC 10132	10	10	76	97	93	99	71	76	66	20	19
	ATCC 35702	7	5	72	103	95	97	74	73	54	18	17
	ATCC 39057	6	5	81	ND	ND	ND	ND	ND	ND	20	21
	NCIMB 6444	24	32	73	78	79	77	68	68	(100)	21	23
	NCIMB 6445	9	10	84	108	98	101	80	80	79	21	12
	NCIMB 8049	6	10	82	108	98	98	77	83	80	21	21
	NCIMB 8052[57]	4	8	78	(100)	101	98	79	81	65	20	20
	NCIMB 8052[Y]	7	8	78	97	(100)	98	79	81	61	22	24
	NCP 193	8	11	82	80	82	84	73	100	65	22	18
	NCP 270	5	4	82	89	83	79	72	(100)	75	18	20
	NCP 271	18	17	81	83	87	84	75	103	72	20	21
	NRRL B466	7	9	79	85	86	86	94	80	76	24	23
	NRRL B591	12	22	84	105	99	101	75	78	72	20	19
	NRRL B592	3	5	76	89	79	81	(100)	73	77	18	19
	NRRL B593	5	4	75	80	77	75	73	75	80	22	19
	NRRL B594	6	8	83	108	102	(100)	79	75	67	20	20
	NRRL B596	18	19	77	89	85	90	98	80	67	22	21
Others	NCP 262	14	26	21	25	22	21	ND	ND	ND	94	19
	NRRL B643	5	12	20	24	19	19	ND	ND	ND	(100)	15
	N1-4	23	25	24	23	23	24	ND	ND	ND	21	(100)
	N1-4081	4	8	26	31	24	26	ND	ND	ND	20	99

^a NCIMB 8052[57] is a direct subculture from a May 1957 culture of NCIMB 8052. NCIMB 8052[Y] is a current laboratory culture of M. Young.

^b ND, not determined.

distinct lineages described by Johnson and Chen (12). The results of DNA reassociation measurements (Table 2) clearly separated the 39 cultures into four groups (Fig. 1). Within each group, the level of DNA sequence similarity was generally 70% or higher, whereas between groups the degree of DNA sequence similarity never exceeded 32%. The results indicated that the cultures belonged to four species (10).

The first group, which contained 17 cultures, was represented by strain ATCC 824, the type strain of *C. acetobutylicum*, and hence comprised this species. This species assignment was supported by the presence in this group of strains DSM 792 and NRRL B527, which are considered equivalent to strain ATCC 824^T. Cultures NCIMB 8052[57] and NCIMB 8052[Y], which exhibited less than 8% DNA sequence similarity to strain ATCC 824^T, were obviously not *C. acetobutylicum* strains. Within this group, only strain NRRL B528 exhibited levels of DNA sequence similarity (84 to 87%) that were significantly lower than those of the other strains in comparison with the reference strains. Thus, authentic strains of *C. acetobutylicum* are genetically very similar.

The second group, which contained 18 cultures, was represented by strain VPI 5481, the type strain of *C. beijerinckii*, and hence comprised this species. Most of the cultures in this group, including NCIMB 8052, have been labeled *C. acetobu-*

FIG. 1. Dendrogram showing levels of DNA relatedness among *C. acetobutylicum*, *C. beijerinckii*, *Clostridium* sp. strains NRRL B643 and NCP 262, and "*C. saccharoperbutylacetonicum*." The average percentages of similarity from Table 2 were clustered by using the unweighted pair group method.

tylicum previously. Among the strains of *C. beijerinckii*, there was a broad range of levels of DNA sequence similarity, and strain NCIMB 6444 exhibited relatively low levels of similarity (as low as 54%) to some strains of *C. beijerinckii*. Therefore, the strains of *C. beijerinckii* are genetically more diverse than the strains of *C. acetobutylicum*.

The third group consisted of *Clostridium* sp. strains NCP 262 (2) and NRRL B643. These two cultures were highly related to each other and belonged to the same species. A search of the cellular fatty acid profiles in the VPI Anaerobic Bacteria Database did not reveal a match between strain NRRL B643 and any presently recognized *Clostridium* species. It has been reported (14) that strain NRRL B643 represents "*Clostridium saccharo-butyl-aceticum-liquefaciens*," an organism used in industrial solvent production from molasses after 1938. Strain NCP 262 was perhaps derived from strain NRRL B643, or both were derived from a common parent strain (16).

The fourth group consisted of *Clostridium* sp. strain N1-4 (8) and its derivative, strain N1-4081 (22). The parent organism was named "*Clostridium saccharoperbutylacetonicum*" (8), but its derivatives have been referred to as *C. acetobutylicum* (22). The DNA reassociation data established that this group is a separate species. "*C. saccharoperbutylacetonicum*" was not included on the Approved Lists of Bacterial Names (24).

Useful phenotypic traits for identification of *C. acetobutylicum*. DNA relatedness, as illustrated in Fig. 1, provides an unambiguous criterion for separation of bacterial species (10, 26). However, measuring levels of DNA reassociation is not practical in many laboratories, and phenotypic traits that correlate with the species established by DNA relatedness remain valuable for proper identification of species. During this study, the two phenotypic traits described below were found to be useful for presumptive identification of *C. acetobutylicum*.

(i) Production of riboflavin in milk medium. Milk is an excellent medium for maintenance of all of the strains used in this study. The stock cultures were incubated at 37°C for 5 days and then stored at 4°C (1). The rate of curd formation was the first useful criterion for differentiation of species. "*C. saccharoperbutylacetonicum*" did not cause curd formation during the first 48 h, whereas the other three species caused curd formation within 24 h, with the strains of *C. acetobutylicum* giving the fastest reaction. After storage at 4°C for about 10 days, a bright yellow color started to appear in milk cultures of all strains of *C. acetobutylicum* but not in any culture of the other three species (data not shown; color photographs of the cultures are available upon request). The color intensity increased with time during the first several weeks and became stable thereafter. This color formation was easier to observe in tube cultures than in bottle cultures because the zone of clear whey was more conspicuous in the tubes. Visible absorption spectra of yellow culture filtrates had an absorption peak at 445 nm (data not shown), which is consistent with the spectrum of riboflavin. Production of riboflavin by *C. acetobutylicum* in low-metal media was once a useful industrial process (27). Based on an ϵ of $11 \text{ mM}^{-1} \text{ cm}^{-1}$ at 445 nm and pH 4.5, the culture filtrates of *C. acetobutylicum* strains contained maximal riboflavin concentrations of 5 to 15 μM . Apparently, milk provides the right conditions for production of riboflavin by *C. acetobutylicum*, and the concomitant appearance of a bright yellow color is the most distinctive phenotypic trait of *C. acetobutylicum*.

(ii) Production of *n*-butanol in TYG medium. We previously reported poor solvent production by *C. acetobutylicum* in TYG medium containing 20 g of glucose per liter (7). The average concentration of *n*-butanol produced by the 17 cultures of *C. acetobutylicum* in TYG medium was 9.0 mM (range, 3.9 to 25 mM). The average concentration of *n*-butanol produced by the

18 cultures of *C. beijerinckii* in the same medium was 48 mM (range, 0.9 to 86 mM). It is interesting that *C. beijerinckii* NCP 270, which is one of the most recently used industrial strains (25), and *C. beijerinckii* NCP 271 produced the highest levels of butanol (75 and 86 mM, respectively) in this sugar-based medium. Strains NCP 262 and NRRL B643 produced 49 and 28 mM *n*-butanol, respectively, whereas strain N1-4 produced 28 mM. Thus, if a culture produces more than 30 mM butanol in TYG medium, it is reasonably certain that the culture is not a *C. acetobutylicum* culture; however, the reverse is not true because solvent-producing clostridia degenerate easily (4, 7, 12). This property is useful for detection of a culture that has been mislabeled or misidentified as *C. acetobutylicum*.

DISCUSSION

Based on DNA relatedness values (this study) and 16S rRNA gene sequences (16), the historical strains, as well as some more recent isolates of solvent-producing clostridia, clearly belong to four species; *C. acetobutylicum* ATCC 824^T, *C. beijerinckii* VPI 5481^T (= ATCC 25752^T), *Clostridium* sp. strain NRRL B643 (or NCP 262), and "*C. saccharoperbutylacetonicum*" N1-4 represent these four groups. The last two groups are each represented by one independent strain, and the historical backgrounds of these two strains have been described by Keis et al. (16). At present, there is insufficient phenotypic information to allow definitive descriptions of the two species encompassing these two strains, although *Clostridium* sp. strain NRRL B643 can be differentiated from the other three groups by phenotypic traits (see below). Further phenotypic characterization of the last two groups of organisms is in progress in the laboratories from which we obtained these strains. Descriptions of these two groups of organisms as two species should not be published until more phenotypic traits, and preferably more strains, are available.

Identification and classification of solvent-producing clostridia have been difficult because these organisms have few phenotypic differences. The solvent-producing clostridia were once classified as the butyl organisms to distinguish them from the true butyric anaerobes (19, 20). In the late 1920s, the species *C. acetobutylicum* was proposed to include strains that produced solvents from starch (19, 28). This use of solvent production as a primary criterion for taxonomy was a source of serious problems because solvent production is an unstable trait whose manifestation changes dramatically when growth conditions change or when strains degenerate (4, 7, 12). The metabolic switch between the acid- and solvent-producing phases, which is accompanied by changes in the culture pH, the profiles of fermentation products, and the profiles of cellular fatty acids, makes the task even more difficult.

When molecular approaches are used, the four species of solvent-producing clostridia can be reliably differentiated. In addition to measurements of DNA relatedness (Fig. 1), the complete sequences of the 16S rRNA genes of representatives of the four groups are available from GenBank under the following accession numbers: *C. acetobutylicum* ATCC 824^T, U16166 and X78070; *C. beijerinckii* DSM 791 (= ATCC 25752^T), X68179; *C. beijerinckii* NCIMB 9362 (= ATCC 25752^T), X68180; *Clostridium* sp. strain NCP 262, U16147 and X78073; and "*C. saccharoperbutylacetonicum*" N1-4, U16122. The 550-nucleotide region between positions 830 and 1383 (*Escherichia coli* numbering) is useful for differentiating these four species (16). Pyrolysis mass spectrometry has been used to separate six strains of solvent-producing clostridia into four groups corresponding to the four species, except that strains NCP 262 and N1-4081 (a deriv-

ative of strain N1-4) were not separated as clearly as the other taxa (29).

C. acetobutylicum and *C. beijerinckii* have been differentiated by the requirement of *C. acetobutylicum* for a fermentable carbohydrate for growth, and a definitive test may be based on the results of this study and the results of Keis et al. (16). The following phenotypic traits should be useful for differentiating the solvent-producing clostridia: *C. acetobutylicum* is susceptible to 10 and 100 ng of rifampin per disc (16) and produces riboflavin to give a bright yellow color in milk; the *Clostridium* sp. represented by strains NRRL B643 and NCP 262 is susceptible to 10 and 100 ng of rifampin per disc (16) but does not produce riboflavin in milk; and *C. beijerinckii* and "*C. saccharoperbutylacetonicum*" are resistant to 10 and 100 ng of rifampin per disc (16) and do not produce riboflavin in milk. A reliable phenotypic trait for differentiating *C. beijerinckii* and "*C. saccharoperbutylacetonicum*" has not been determined yet. Nevertheless, all laboratory strains of solvent-producing *C. beijerinckii* have been correctly identified by DNA relatedness data.

ACKNOWLEDGMENTS

We are grateful to the investigators listed in Table 1 for providing cultures, to the late W. E. C. Moore for analyzing the cellular fatty acid profile of strain NRRL B643, and to A. Ismael for analyzing the visible absorption spectra of the culture supernatants. We are saddened by the deaths of John L. Johnson and W. E. C. Moore shortly after the completion of this study.

This study was supported by U.S. Department of Energy grant DE-FG05-85-ER13368 and by the Cooperative State Research Service, U.S. Department of Agriculture, under project 6129960.

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