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Short Communication

Assignment of the six solvent-producing *Clostridium* spp. to *Clostridium beijerinckii* based on DNA-DNA reassociation

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Industrial production of acetone and butanol by solvent-producing *Clostridium* spp. strains was among the first large-scale fermentation processes developed (Jones and Woods, 1986). Commercial production of acetone and butanol by fermentation began with the Weizmann process (Jones and Woods, 1986; Walton and Martin, 1979). The Weizmann organism was used extensively to produce acetone from maize mash during World War I. From 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. For patent purposes, each new strain was assigned a novel species name, but the nomenclature adopted was not systematic and was applied in a haphazard manner (Ross, 1961; Ryden, 1958).

In 1926, McCoy et al. performed a comparative study of 11 starch-utilizing strains (McCoy et al., 1926). These investigators concluded that small phenotypic variations occurred in these strains, but all 11 strains should be grouped in a single species named *C. acetobutylicum* (McCoy et al., 1926). Weyer and Rettger (1927) also performed a comparative study of six different starch-utilizing, solvent-producing strains belonging to *C. acetobutylicum* and selected one of these strains, ATCC 824, which was isolated from Connecticut garden soil in 1924, as the type strain of the species (Weyer and Rettger, 1927). Thus almost all historic strains of solvent-producing clostridia available from major culture collections have been listed as *C. acetobutylicum*.

Recently, several strains of *C. acetobutylicum* were subjected to detailed biochemical, physiological, and genetic analysis, and these results indicated that some of these strains were actually *C. beijerinckii* based on the DNA-DNA reassociation method (Johnson et al., 1997). Earlier results of 16S rDNA analysis showed that *C. acetobutylicum* was closely related to *C. beijerinckii* (Collins et al., 1994; Hutson et al., 1993; Lawson et al., 1993), but these studies used a culture of *C. acetobutylicum* that was later identified as *C. beijerinckii*.

On the other hand, many solvent-producing bacteria that have characters different from those of *C. acetobutylicum* were isolated and named. For example, "*C. butanologenum*" (Asai and Haruta 1943) JCM 7833, "*C. butyricum* var. *convexa*" (Sugama) JCM 7839, "*C. isopropylicum*" (Sugama) JCM 7844, 7845, and "*C. pectinolyticum*" (Prevot 1957) JCM 7847 were newly isolated, assigned a novel species name, and added to culture collections of the Japan Collection of Microorganisms (JCM) and Institute of Applied Microbiology (IAM). These invalid species are solvent-producing clostridia with different characters, but their taxonomic positions are not clear.

These solvent-producing clostridia were characterized, and 16S rDNA sequences were used to determine their taxonomic positions. To identify strains of a species, we used DNA-DNA reassociation to analyze five solvent-producing clostridia.

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The bacterial strains used in this study were "*C. butanologenum*" JCM 7833, "*C. butyricum* subsp. *convexa*" JCM 7839, "*C. isopropylicum*" JCM 7844, "*C. isopropylicum*" JCM 7845, "*C. pectinolyticum*" JCM 7847, *C. beijerinckii* JCM 1390^T, and *C. acetobutylicum* JCM 1419^T. All bacterial strains were cultivated for 2 days at 37°C on EG agar [premixed EG agar (Eiken Chemical Co., Ltd., Tokyo) supplemented with 5% horse blood and containing beef extract 3 g, yeast extract 5 g, peptone 10 g, glucose 1.5 g, L-cysteine HCl 0.5 g, L-cystine 0.2 g, Na_2HPO_4 4 g, soluble starch 0.5 g, Tween 80 0.5 g, silicone 0.5 g, and agar 15 g in 1,000 ml, pH 7.7] in an anaerobic chamber. Acid production from carbohydrates, assimilation of organic acids, nitrate reduction, and hydrolysis of starch and gelatin were tested (Holdeman et al., 1977; Kaneuchi et al., 1976). DNA was isolated as described by Saitou and Nei (Saitou and Nei, 1987). DNA base composition was estimated by high-performance liquid chromatography (HPLC) (Kaneko et al, 1986; Tamaoka and Komagata, 1984). The 16S rRNA gene was amplified by the PCR method by using prokaryotic 16S rDNA universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR was performed with a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.), using 30 cycles consisting of denaturation at 94°C for 60 s, primer annealing at 55°C for 150 s, and primer extension at 72°C for 150 s (with 30 s added per cycle). Sequencing was performed by using an ALFred AutoCycle Sequencing Kit (Pharmacia Biotech, Tokyo, Japan) with an ALFexpress DNA sequencer (Pharmacia Biotech). Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura and Ohta, 1972), and phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987). The topology of the trees was evaluated by performing a bootstrap analysis of the sequence data with the CLUSTAL W software (Willems and Collins, 1996). The sequences determined in this study have been deposited in the DDBJ data library under the following accession numbers: AB020187 for *C. beijerinckii* JCM 7833, AB020188 for *C. beijerinckii* JCM 7839, AB020189 for *C. beijerinckii* JCM 7844, AB020190 for *C. beijerinckii* JCM 7845, and AB020191 for *C. beijerinckii* JCM 7847.

The 16S rDNA sequences of the solvent producing clostridial species, "*C. butanologenum*" JCM 7833, "*C. butyricum* var. *convexa*" JCM 7839, "*C. isopropylicum*" JCM 7844, 7845, and "*C. pectinolyticum*" JCM 7847, which were differentiated from *C. acetobutylicum* by phenotypic characters, although their taxonomic position was not clear, were determined. More than 1,400 bases of the 16S rDNA sequences (positions 28 to 1492; *Escherichia coli* numbering system) of the five solvent-producing clostridia were determined, and these sequences have been deposited in the DDBJ database. Figure 1 shows a phylogenetic tree based on calculated K_{nuc} values created using our sequences and sequences obtained from databases. It is difficult to differentiate *C. acetobutylicum* and *C. beijerinckii* by using phenotypic characters; thus many solvent-producing clostridia were misidentified as *C. acetobutylicum* for a long time. In this study, the 16S rRNA sequence was determined and used to resolve the taxonomic position of the organisms under investigation.

Five solvent-producing clostridia with invalid names, as well as *C. acetobutylicum* and *C. beijerinckii*, were characterized. All these five solvent-producing clostridia with invalid names and *C. beijerinckii* produced acid from arabinose, fructose, galactose, and lactose and did not produce acid from rhamnose, ribose, or dulcitol. Esculin hydrolysis, starch hydrolysis, and gas formation were positive, and bile stimulation, indole production, nitrate reduction, H_2S production, motility, gelatin liquefaction, and growth at 5.0 and 6.5% NaCl were negative. On the other hand, with *C. acetobutylicum,* acid production from sugars was similar to that of *C. beijerinckii*, but it could be differentiated by acid production from melibiose, raffinose, melezitose, and inulin or by H_2S production (Table 1). The DNA base composition of these five solvent-producing clostridia with invalid names, *C. acetobutylicum* and *C. beijerinckii* was 30.7–31.8, 33.4, and 31.2 mol% G+C, respectively (Table 2). The levels of DNA-DNA relatedness among these five solvent-producing clostridia with invalid names and *C. beijerinckii* ranged from 70 to 126% (Table 2).

The result of 16S rDNA sequencing shows that these five clostridia were taxonomically very close to *C. beijerinckii*. Based on the levels of DNA relatedness, it is clear that these five strains are actually *C. beijerinckii*. Physiological and biochemical characteristics shown in Table 1 indicate that differentiation of *C. acetobutylicum* and *C. beijerinckii* was difficult, but some sugar fermentation patterns are characteristic. *C. acetobutylicum* did not produce acid from melibiose, raffinose, melezitose, or inulin, but *C. beijerinckii* fermented these sugars. H_2S production was also clear; *C. acetobutylicum* produced H_2S, but *C. beijerinckii* did not.

Numerous saccharolytic solvent-producing clostridia were isolated and patented from the mid-1930s. *C. acetobutylicum* and other solvent-producing clostridia have been studied concerning their industrial utility, but phenotypic characterization was not performed in detail, and it was difficult to identify them at the species level until now.

In the present study, the taxonomic position of sol-

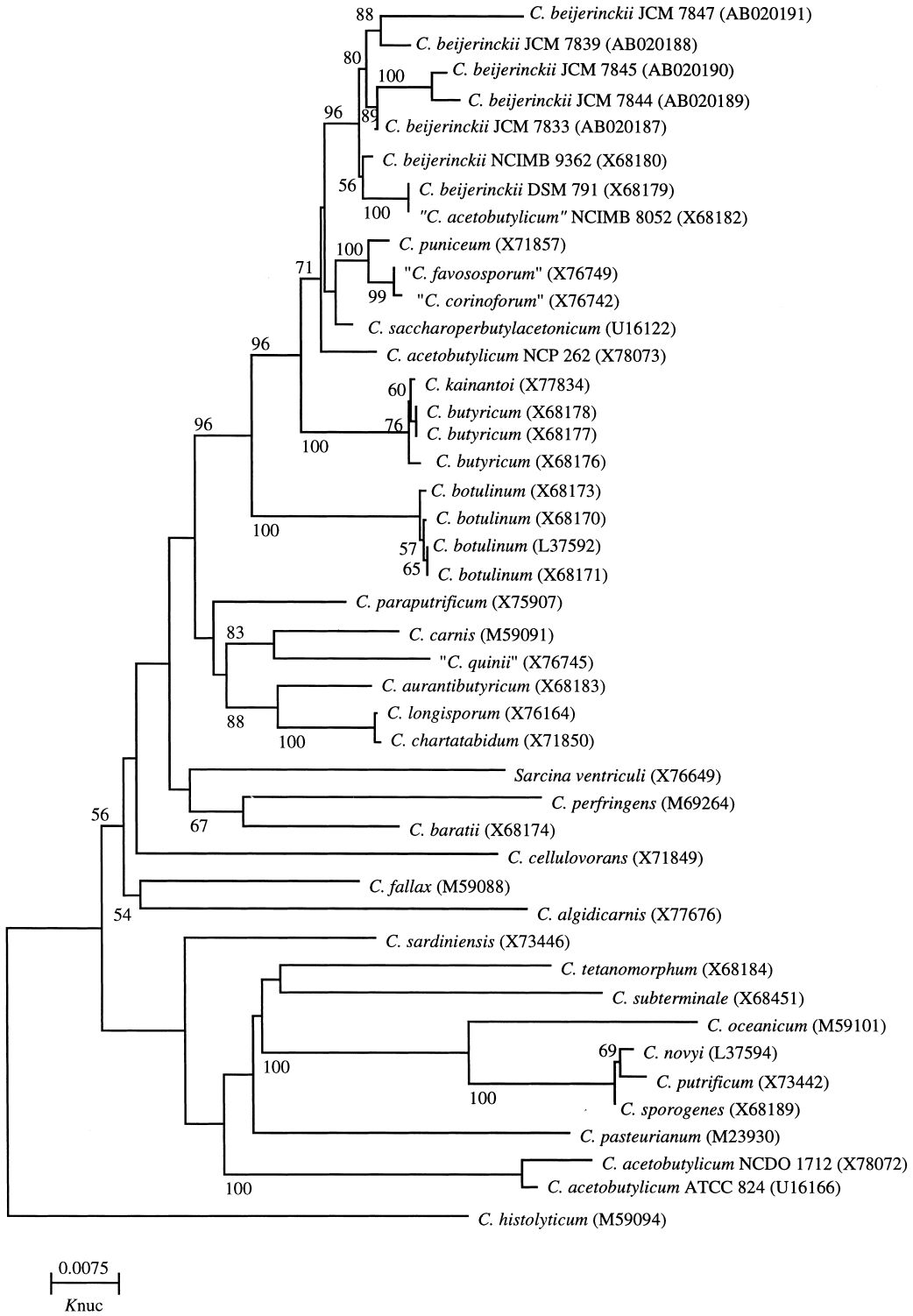

Fig. 1. Unrooted phylogenetic tree of invalid clostridial strains and close relatives derived from 16S rDNA sequences.
The tree was created by using the neighbor-joining method and K_{nuc} values. The numbers on the tree indicate bootstrap values for the branch points. The sequence data for the species besides strains *C. beijerinckii* JCM 7833 (AB020187), *C. beijerinckii* JCM 7839 (AB020188), *C. beijerinckii* JCM 7844 (AB020189), *C. beijerinckii* JCM 7845 (AB020190), and *C. beijerinckii* JCM 7847 (AB020191) designations were obtained from the database. *C.*: *Clostridium*.

Table 1. Physiological and biochemical characteristics of strains of solvent-producing bacteria.

Characteristics	*C. acetobutylicum* JCM 1419^T	*C. beijerinckii* JCM 1390^T	"*C. butanologenum*" JCM 7833	"*C. butyricum* subsp. *convexa*" JCM 7839	"*C. isopropylicum*" JCM 7844	"*C. isopropylicum*" JCM 7845	"*C. pectinolyticum*" JCM 7847
L-Arabinose	+	+	+	+	+	+	+
D-Xylose	+	w	+	+	+	+	+
Rhamnose	−	−	−	−	−	−	−
Ribose	−	−	−	−	−	−	−
Glucose	+	+	+	+	w	w	+
Mannose	+	w	+	+	−	−	+
Fructose	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+
Sucrose	+	w	+	+	−	−	+
Maltose	+	w	+	+	w	w	+
Cellobiose	+	w	+	+	−	−	+
Lactose	+	+	+	+	+	+	+
Trehalose	−	w	−	w	−	−	+
Melibiose	−	w	+	+	+	+	+
Raffinose	−	w	+	+	+	w	+
Melezitose	−	w	+	w	+	+	+
Starch	+	−	+	+	+	w	w
Glycogen	+	−	+	+	+	+	+
Inulin	−	w	+	+	+	+	+
Mannitol	+	−	+	+	+	+	−
Sorbitol	−	−	+	w	w	w	−
Inositol	−	+	+	+	+	+	−
Dulcitol	−	−	−	−	−	−	−
Esculin	−	−	+	w	w	w	−
Salicin	−	−	+	+	w	w	+
Amygdalin	−	−	+	w	w	w	w
Esculin hydrolysis	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+
Gas formation	+	+	+	+	+	+	+
Bile stimulate	−	−	−	−	−	−	−
Indole production	−	−	−	−	−	−	−
Nitrate reduction	−	−	−	−	−	−	−
H_2S production	+	−	−	−	−	−	−
Motility	−	−	−	−	−	−	−
Gelatin liquefaction	−	−	−	−	−	−	−
Growth at 5.0% NaCl	−	−	−	−	−	−	−
Growth at 6.5% NaCl	−	−	−	−	−	−	−

Symbols: +, positive reaction; −, negative reaction; w, weak reaction.

Table 2. DNA base composition and levels of DNA-DNA relatedness among clostridial strains.

Strain	(mol%)	% DNA-DNA reassociation with						
		JCM 7833	JCM 7839	JCM 7844	JCM 7845	JCM 7847	JCM 1390	JCM 1419
"*C. butanologenum*" JCM 7833	30.7	100	74	93	91	70	85	8
"*C. butyricum* subsp. *convexa*" JCM 7839	31.8	76	100	70	70	83	77	8
"*C. isopropylicum*" JCM 7844	31.3	126	98	100	105	85	99	6
"*C. isopropylicum*" JCM 7845	30.3	115	96	96	100	81	93	5
"*C. pectinolyticum*" JCM 7847	31	94	99	85	85	100	81	10
C. beijerinckii JCM 1390	31.2	87	93	91	92	83	100	10
C. acetobutylicum JCM 1419	33.4	10	11	13	12	12	10	6

vent-producing clostridia was determined by using both the 16S rDNA sequence and the levels of DNA-DNA relatedness. Five invalid species of solvent-producing clostridia examined in this study are assigned to *C. beijerinckii*.

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