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Acetone, Isopropanol, and Butanol Production by *Clostridium beijerinckii* (syn. *Clostridium butylicum*) and *Clostridium aurantibutyricum*

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Thirty-four strains representing 15 species of anaerobic bacteria were screened for acetone, isopropanol, and *n*-butanol (solvent) production. Under our culture conditions, several strains of *Clostridium beijerinckii* and *C. aurantibutyricum* produced at least 40 mM *n*-butanol (*C. acetobutylicum* strains produced up to 41 mM *n*-butanol under similar conditions). Both solvent-producing and non-solvent-producing strains of *C. beijerinckii* have high DNA homology with a reference strain of *C. beijerinckii*. Strains labeled "*Clostridium butylicum*" are phenotypically similar to *C. beijerinckii* and showed at least 78% DNA homology to a reference strain of *C. beijerinckii*. Therefore, these "*C. butylicum*" strains are members of *C. beijerinckii*. An earlier DNA homology study has shown that *C. beijerinckii*, *C. aurantibutyricum*, and *C. acetobutylicum* are distinct species.

Since the 1950s, acetone, *n*-butanol, and isopropanol have been produced almost exclusively from petrochemicals. But because of changing economics, the industrial production of these chemicals or "solvents" by fermentation is being evaluated as an alternative to the synthetic processes (12, 14, 16–19). Maximum efficiency and flexibility of solvent fermentation will in part depend upon the availability of a selection of organisms of various phenotypic and genotypic properties. Historically, two clostridial species, *Clostridium acetobutylicum* and "*Clostridium butylicum*," have been used in or developed for solvent fermentation. However, other fermentative anaerobes also produce some solvents during growth (8). We are not aware of studies which compare quantitatively solvent production by these other fermentative organisms with that by *C. acetobutylicum* or "*C. butylicum*." In the present study we found that certain anaerobic species compare favorably with *C. acetobutylicum* and "*C. butylicum*" in solvent-forming ability, and we present additional findings which bear on the taxonomic status of "*C. butylicum*."

(A portion of this work was previously presented [Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, O13, p. 198].)

Based on the species descriptions provided in the Virginia Polytechnic Institute and State University (VPI) *Anaerobe Laboratory Manual* (8) and additional biochemical properties, we chose strains representing 15 species of anaerobic bacteria from the departmental culture collection and quantitated their solvent production under standardized growth conditions. All species fermented glucose. Results were compared with those of seven strains of *C. acetobutylicum* and of two strains labeled "*C. butylicum*" originally developed for industrial fermentation; the latter strains were obtained from the Northern Regional Research Center, U.S. Department of Agriculture, Peoria, Ill. (hereafter referred to as the NRRL strains). All cultures used in this study were incubated statically at 35°C and were mixed daily by inversion. Media were prepared under a nitrogen atmosphere (8). To revive lyophilized preparations of each strain, the dried cells were inoculated into chopped meat-carbohydrate medium (8) and incubated for 3 to 4 days. Lyophilized cultures of *C. acetobutylicum* were more reliably revived in a potato medium (P. T. Bui, Ph.D. thesis, Purdue University, W. Lafayette, Ind., 1968) supplemented with glucose (5 g/liter) and $CaCO_3$. (One strain of *C. acetobutylicum* [VPI 13698] was revived from spores; whether that might have affected the test result has not been determined.) Strains were then inoculated (5% vol/vol) into a peptone-yeast extract-glucose medium of the following composition (grams per liter of distilled water): tryptone (Difco, Detroit, Mich.), 1.0; yeast extract (Difco), 5.0; glucose, 20.0; Na_2SO_4, 0.18; K_2HPO_4, 3.48; biotin, 0.01; *p*-aminobenzoic acid, 0.01; cysteine-hydrochloride, 0.5. The medium also contained 1 ml of a mineral stock

TABLE 1. Solvent production by selected anaerobes in a peptone-yeast extract-glucose medium[a]

Species	Strain		Solvent produced (mM)[b]		
	VPI no.	Other no.[c]	Acetone	Isopropanol	*n*-Butanol
C. beijerinckii[d]	5481 (type)	ATCC 25752	6.0	—	67.9
	4635	—	10.2	—	69.6
	2697	—	8.7	—	55.2
	4419	ATCC 11914	2.3	—	17.0
	2408	—	—	—	4.4
	4213	ATCC 14949	—	—	—
	2983	—	—	—	—
	2968	—	—	9.8	44.8
	2982	—	—	1.6	41.3
"*C. butylicum*"	2776	—	—	—	—
	2844	ATCC 14823	—	—	—
	13436	NRRL B-592	19.0	—	60.4
	13437	NRRL B-593	—	8.0	61.7
C. aurantibutyricum[d]	4633 (type)	ATCC 17777	20.5	4.5	45.4
	10789	NCIB 10659	14.5	10.0	42.4
C. acetobutylicum	2673	McClung 633	22.3	—	41.3
	13697	ATCC 4259	6.2	—	19.5
	13698	NRRL B-527 (←ATCC 824)	6.8	—	17.1
	13693	ATCC 8529	1.7	—	4.5
	2676	McClung 635	1.1	—	3.3
	13696 (type)	ATCC 824	—	—	2.4
	13692	ATCC 10132	—	—	—
C. pasteurianum[d]	2670	—	—	—	12.2
C. sporogenes	6014	—	—	—	18.7
C. cadaveris	6834A	—	—	—	11.2

[a] The following VPI strains produced less than 1 mM solvent products: *C. barkeri* 5359 (ATCC 25849 [type strain]), *C. fallax* 6008, *C. glycolicum* 3284, *C. bifermentans* 2414, *C. subterminale* 5360, *Megasphaera elsdenii* 6135, *Fusobacterium mortiferum* 8679, *Eubacterium combesii* 5839, *Eubacterium limosum* 5847.

[b] —, Less than 1 mM product formed.

[c] ATCC, American Type Culture Collection, Rockville, Md.; NRRL, Northern Regional Research Center, U.S. Department of Agriculture, Peoria, Ill.; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland; McClung, L. S. McClung, Indiana University, Bloomington.

[d] For additional strain designations, see reference 5.

solution per liter (P. T. Bui, Ph.D. thesis) which contained (per liter): $NaMoO_4 \cdot 2H_2O$, 2.4 g; $CoCl_2 \cdot 6H_2O$, 0.24 g; $CaCl_2 \cdot 2H_2O$, 1.5 g; $FeCl_3 \cdot 6H_2O$, 27 g; H_2SO_4, 28 ml; $CuSO_4 \cdot 5H_2O$, 0.25 g; $ZnSO_4 \cdot 7H_2O$, 0.29 g; $MnSO_4 \cdot H_2O$, 1.7 g; $MgSO_4$, 12 g. The medium was adjusted to pH 6.8 with HCl before autoclaving and was dispensed 7 ml per tube. After 6 days of incubation, solvent production was quantitated in culture supernatants by using a Gow-Mac series 750 FID gas chromatograph (Gow-Mac Instrument Co., Bridgewater, N.J.). For *n*-butanol determinations, a stainless-steel column (1.2 m by 3.2 mm) containing Chromosorb 101 (80/100 mesh; Supelco, Inc., Bellefonte, Pa.) was used. For acetone and isopropanol determinations, a stainless-steel column (1.8 m by 3.2 mm) containing Porapak Q (100/120 mesh; Supelco, Inc.) was used. In each case, the column oven temperature was 120°C, the injector and detector temperatures were 160°C, nitrogen was used as the carrier gas at a flow rate of 24 ml/min, and isobutanol was used as an internal standard.

Acetone, butanol, and isopropanol production by the strains tested is indicated in Table 1. Strains from the VPI collection that produced at least 40 mM *n*-butanol under these conditions are members of *C. beijerinckii* (five of nine strains) and *C. aurantibutyricum* (two of two strains). Strains of *C. aurantibutyricum* produced higher concentrations of acetone and isopropanol in relation to *n*-butanol than strains of *C. beijerinckii*. Two strains from the VPI collection labeled "*C. butylicum*" (VPI 2776 [= McClung 1186] and VPI 2844 [= McClung 629, McCoy 79]) did not produce appreciable concentrations of solvents. The two NRRL strains (B-592 [McCoy A-39 ← Fernbach] and B-593 [McCoy A-21 ← Beijerinck]) of "*C. butylicum*" tested produced at least 60 mM *n*-butanol under these conditions. The concentration of *n*-butanol produced by seven strains of *C. acetobutylicum* varied from <1 to 41 mM.

The product yields obtained in this study probably represent submaximal estimates of solvent-producing ability. First, the physiological

states of inocula were not precisely controlled, which could have affected the product yield of some strains more than others. Second, solvent levels in cultures of *C. acetobutylicum* and "*C. butylicum*" are dependent upon the concentration of carbohydrate present in the growth medium (4). "*C. butylicum*" NRRL B-592 when grown in 800-ml batch cultures in the medium described here but containing 6% sucrose instead of 2% glucose gave approximately twice the solvent yield that is indicated for this strain in Table 1. It is likely that other strains would display similar behavior. We conclude that several strains of *C. aurantibutyricum* and *C. beijerinckii* are potentially useful for the development of acetone-butanol fermentation.

Butyric acid-producing clostridia have been subdivided into those which display "butyric" versus "butylic" fermentation, characterized by the predominant production of acids or acids plus neutral products, respectively (13). These organisms often share many phenotypic properties (3), hindering the classification and identification of new isolates. The genetic relationships which exist between the various butyric acid-producing clostridia were, in part, established by the DNA homology and cell wall studies of Cummins and Johnson (5). However, this study did not include strains labeled "*C. butylicum*." Despite the appearance of "*C. butylicum*" in current literature and its listing in various collections such as the American Type Culture Collection, "*C. butylicum*" is no longer a recognized species nor is it considered a synonym for an existing species. The last complete description of the organism appeared in the 7th edition of *Bergey's Manual* (3). We examined the phenotypic characteristics (about 80 traits) of "*C. butylicum*" NRRL B-592, NRRL B-593, VPI 2776, and VPI 2844 and found that they match closely the current description of *C. beijerinckii* (3, 8). We then measured the degree of DNA homology between the "*C. butylicum*" strains and a reference strain of *C. beijerinckii* (VPI 2983) by the S1 nuclease method (10). The two non-solvent-producing strains deposited in the VPI collection as "*C. butylicum*" and the solvent-producing NRRL strains of "*C. butylicum*" showed high DNA homology with *C. beijerinckii* VPI 2983. The DNA homology between these four "*C. butylicum*" strains and *C. beijerinckii* VPI 2983 are as follows: VPI 2776, 83%; VPI 2844 (= ATCC 14823), 87%; NRRL B-592, 78%; NRRL B-593, 84%. In comparison, the DNA homology (5) between seven other strains of *C. beijerinckii* and strain 2983 are as follows: VPI 5481 (= ATCC 25752, the type strain), 91%; VPI 4635, 92%; VPI 2697, 80%, VPI 4419 (= ATCC 11914), 79%, VPI 2408, 79%, VPI 4213 (= ATCC 14949), 93%, VPI 2968, 87%. Homology values greater than 70% indicate genetic relatedness at the species level (9). We conclude that the four "*C. butylicum*" strains examined in this study are properly designated *C. beijerinckii*. Although "*C. butylicum*" would have priority as the species designation over *C. beijerinckii*, "*C. butylicum*" did not appear on the 1980 "Approved Lists of Bacterial Names" (5), apparently through an oversight. Genetic relatedness between "*C. butylicum*" and *C. beijerinckii* is not surprising in view of the description provided in the 7th edition of *Bergey's Manual* (3); indeed, "*C. butylicum*" and *C. beijerinckii* ferment a similar array of carbohydrates. The two "species" were differentiated by the inability of *C. beijerinckii* to ferment starch, whereas "*C. butylicum*" ferments starch of potato mash but not of maize mash. However, many strains of *C. beijerinckii* are found to ferment starch (8).

It should be noted that solvent production is not a stable trait. Strains of *C. acetobutylicum* and "*C. butylicum*," which produced significant amounts of solvent when first isolated, have been reported to undergo degenerative processes which cause the loss of solvent-producing capacity (e.g., reference 11). This is illustrated by two of the currently available strains of "*C. butylicum*" (Table 1). The *C. beijerinckii* strains in the VPI collection were not isolated originally for solvent production; nevertheless, they also include solvent-producing and non-solvent-producing strains. Therefore, solvent production is not a reliable criterion for their identification and classification. The designation of "*C. butylicum*" strains as *C. beijerinckii* does not involve any new effort to group solvent-producing and non-solvent-producing organisms into one species; it is the selection between two existing names of an appropriate one for a group of organisms sharing many phenotypic traits and showing a high degree of DNA homology.

Although known to be genetically distinct (5), *C. acetobutylicum* and *C. beijerinckii* are separated by few phenotypic characteristics, the most notable being that *C. acetobutylicum* shows an absolute requirement for a fermentable carbohydrate as a growth substrate (8). Current descriptions (8) indicate that *C. aurantibutyricum* can be distinguished from *C. acetobutylicum* and *C. beijerinckii* by gelatin liquefication and the production of a lipase activity, in addition to DNA homology tests (5).

The physiological factors which are causally related to the onset of acetone-butanol production are being actively investigated in several laboratories. Recent reports suggest that a low pH (pH 5.2 or below) and an elevated concentration of acetate or butyrate or both are required for the onset of acetone-butanol production in *C.*

acetobutylicum (1, 2, 6, 7). We have chosen one strain of *C. beijerinckii* (VPI 13436 or NRRL B-592) for further studies on the cellular control of acetone-butanol fermentation. When the batch fermentation was carried out without pH control, the biphasic phenomenon or the pH break was observed with a pH of 5 reached before butanol was produced. However, we repeatedly obtained butanol in concentrations above 50 mM in fermentations in which the pH was maintained at 6.8. Therefore, neither a change in culture pH nor an acidic pH (below 5) is obligatory for the onset of butanol production in *C. beijerinckii*. A detailed description of the study of physiological control of acetone-butanol fermentation will be published separately.

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