APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1984, p. 573–576
0099-2240/84/090573-04$02.00/0

Vol. 48, No. 3

Formation of *n*-Butanol from D-Glucose by Strains of the "*Clostridium tetanomorphum*" Group

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Received 15 May 1984/Accepted 11 June 1984

A clostridial strain has been isolated that produced *n*-butanol, ethanol, butyrate, and acetate as major fermentation products from glucose but no acetone. At a pH of 6.6, *n*-butanol was formed by this microorganism only during growth. On the basis of its physiological characteristics and DNA-DNA homology data, the strain was assigned to the "*Clostridium tetanomorphum*" group (S. Nakamura, I. Okado, T. Abe, and S. Nishida, J. Gen. Microbiol. 113:29–35, 1979). All members of this group were shown to produce *n*-butanol from glucose as the major fermentation product, whereas *C. cochlearium* produced it in only minor amounts.

In the course of experiments aimed at the isolation of new strains of *Clostridium acetobutylicum*, an isolate was obtained that produced *n*-butanol and ethanol but neither acetone nor isopropanol. This organism, designated strain MG1, was characterized and found to be identical with one strain (DSM 665) of a group of strains for which Nakamura et al. (17) had applied the name "*C. tetanomorphum*." The ability to produce *n*-butanol from glucose in large quantities has been found to be an additional characteristic of the strains of this group.

MATERIALS AND METHODS

Bacterial strains. Strain MG1 was isolated from soil taken from the rhizosphere of bean roots. *Escherichia coli* K-12 (DSM 498) and B (DSM 613) were obtained from the German Collection of Microorganisms, Göttingen, Federal Republic of Germany. The clostridial strains belonging to either the *C. cochlearium* or the "*C. tetanomorphum*" group (17) were obtained from the National Collection of Type Cultures, London, England, and the German Collection of Microorganisms. The strains are listed in Table 1.

Isolation and culture methods. Soil samples were taken from potato fields and the rhizosphere of bean roots and roots of other leguminous plants by the method of Calam (6a). The samples were pasteurized by heating for 10 min at 80°C, and samples were inoculated into phosphate-limited medium containing the following ingredients in 1 liter of distilled water: Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 10 g; yeast extract, 6 g; Tris-hydrochloride, 6.057 g; maleic anhydride, 4.903 g; $MgSO_4 \cdot 7H_2O$, 2.46 g; $CoCl_2 \cdot 6H_2O$, 11.9 mg; resazurin, 1 mg; and sodium thioglycolate, 0.5 g. The pH was adjusted to 6.5. Glucose was sterilized separately as a stock solution (20 to 60% [wt/vol]) and added to the autoclaved medium to a final concentration of 2%. To remove phosphate from the complex components of the medium, 10% (wt/vol) solutions of them were treated with 9 g of $Ca(OH)_2$ per liter while the pH was kept constant with concentrated hydrochloric acid. The solutions were centrifuged at 10,000 × *g* for 20 min, and the clear supernatants were used for medium preparation. After incubation for 3 days, the cultures were transferred to phosphate-carbonate medium containing the following ingredients in 1 liter of distilled water: glucose · H_2O, 20 g; Trypticase peptone, 10 g; yeast extract, 6 g; KH_2PO_4, 3.38 g; $NaHCO_3$, 4 g; $MgSO_4 \cdot 7H_2O$, 2.46 g; $CoCl_2 \cdot 6H_2O$, 11.9 mg; resazurin, 1 mg; and sodium thioglycolate, 0.5 g (pH 6.5). $NaHCO_3$ was added to the autoclaved medium from a 5% (wt/vol) stock solution. Peptone-yeast extract-glucose medium was composed like phosphate-carbonate medium, except that 1 g of KH_2PO_4 and 1 g of K_2HPO_4 were used and no $NaHCO_3$ was added. For the anaerobic preparation of media and solutions, the techniques of Hungate (13) as modified by Bryant (5) were applied. The media were autoclaved for 20 min at 121°C in tightly sealed glass tubes or bottles with rubber stoppers. For growth on plates 1.8% agar was added to Reinforced Clostridial Medium (Oxoid Ltd., London, England). The plates were incubated in anaerobic jars at 37°C.

Stock cultures of the new isolate were made with phosphate-carbonate medium containing 2% (wt/vol) lactose as substrate, because it was found that "*C. tetanomorphum*" sporulated rapidly on this substrate.

Batch culture experiments at a constant pH were done in a Biostat M fermentor (B. Braun Melsungen AG, Federal Republic of Germany). The pH was kept constant with 2 N KOH.

The optical density was measured at 578 nm in a PL4 spectrophotometer (Zeiss, Oberkochen, Federal Republic of Germany). The light path was 1 cm. During growth experiments in glass tubes (1-cm diameter), the optical density was measured at 600 nm in a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.).

Biochemical tests. For strain characterization, the procedures recommended by Holdeman et al. (12) were used.

Determination of DNA base composition and DNA-DNA homology. After isolating the DNA by the method of Marmur (15), the guanine-plus-cytosine (G+C) content (moles percent [mol%]) of the DNA was determined from its thermal denaturation temperature (16). The DNA-DNA hybridization was measured from DNA renaturation rates (8, 9) with a Gilford spectrophotometer 250 with a thermoprogrammer 2527. DNA of *E. coli* K-12 (51.7 mol%G+C) and *E. coli* B (51.0 mol%G+C) were used as reference.

Microscopy. Cultures were regularly examined under a Zeiss light microscope. For better characterization, the cells were examined in a Philips electron microscope EM 301 after negative staining (20).

Determination of substrates and products. Glucose was determined by the glucose test system (E. Merck AG, Darmstadt, Federal Republic of Germany). Phosphate was

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TABLE 1. *Clostridium* strains used in this study

Group and species name as received[a]	Strain (source)
C. cochlearium	
C. cochlearium	DSM 666
C. cochlearium	DSM 667
C. cochlearium	DSM 1285^T (=ATCC 17787^T)
C. cochlearium	3496A (DSM; received from G. Schallehn[b]
C. cochlearium	E4222 (DSM; received from G. Schallehn)
C. lentoputrescens	DSM 2153^T (=ATCC 17794^T)
"*C. tetanomorphum*"	NCTC 2909
"*C. tetanomorphum*"	
"*C. tetanomorphum*"	MG1 (isolated from soil)
Clostridium sp.	DSM 528 (= ATCC 15920)
Clostridium sp.	DSM 665 (= NCTC288)
"*C. tetanomorphum*"	NCTC 500
"*C. tetanomorphum*"	NCTC 543

[a] Group described by Nakamura et al. (17). Names in quotation marks are not included in the *Approved Lists of Bacterial Names* (18).
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measured colorimetrically as molybdate-vanadate complex (Boehringer, Mannheim, Federal Republic of Germany). Soluble products such as *n*-butanol, acetone, ethanol, butyrate, acetate, acetoin, isopropanol, and propionate were determined by gas chromatography. The sample was acidified with 2 N HCl, and 1 μl was injected into a Perkin-Elmer 3920 gas chromatograph equipped with a flame ionization detector. The 2-m glass column was packed with Chromosorb 101 (80/100 mesh). The column temperature was 150°C, the injector temperature was 200°C, and the detector temperature was 220°C. N_2 was used as carrier gas. Analysis of the data was done with an Autolab System IV integrator (Spectra Physics, Santa Clara, Calif.). Concentrations were calculated with isobutanol as an internal standard.

Chemicals. All chemicals used were obtained from Merck AG except the following: glucose, Fluka AG, Buchs, Switzerland; Chromosorb 101, Johns-Monville, Denver, Colo.; resazurin, Serva, Heidelberg, Federal Republic of Germany.

RESULTS

Isolation of strain MG1 and fermentation characteristics. Soil samples were pasteurized and incubated in a glucose-containing medium for 3 days at 37°C. The products formed were determined by gas chromatography. Dilutions of enrichment cultures that produced *n*-butanol were streaked onto agar plates, and various strains were isolated. One of the strains (MG1) obtained did not form any acetone. The solvents produced by strain MG1 and other well-known solvent producers during growth on a peptone-yeast extract-glucose medium are summarized in Table 2. Data from George et al. (11) are included for comparison. Acetone was readily detectable among the products formed by all strains except MG1. The same was true for acetoin, which was produced by *C. acetobutylicum*, *C. beijerinckii*, and *C. aurantibutyricum* in small amounts but not by strain MG1.

TABLE 2. Solvent production by various *n*-butanol-producing *Clostridia* species in peptone-yeast extract-glucose medium containing 2% (wt/vol) glucose

Species	Strain	Solvents produced (mM)			
		Acetone	Isopropanol	*n*-Butanol	Ethanol
C. acetobutylicum	DSM 792	14.0	—[a]	30.2	5.0
C. acetobutylicum[b]	VPI 2673	22.3	—	41.3	ND[c]
C. beijerinckii	DSM 791	8.3	—	44.0	3.3
C. beijerinckii[b]	ATCC 25752	6.0	—	67.9	ND
C. aurantibutyricum	DSM 793	12.3	—	27.1	4.1
C. aurantibutyricum[b]	ATCC 17777	20.5	4.5	45.4	ND
Clostridium sp.	MG1	—	—	47.1	42.7

[a] —, Limit of detection was 0.2 mM.
[b] Results from George et al. (11).
[c] ND, Not determined.

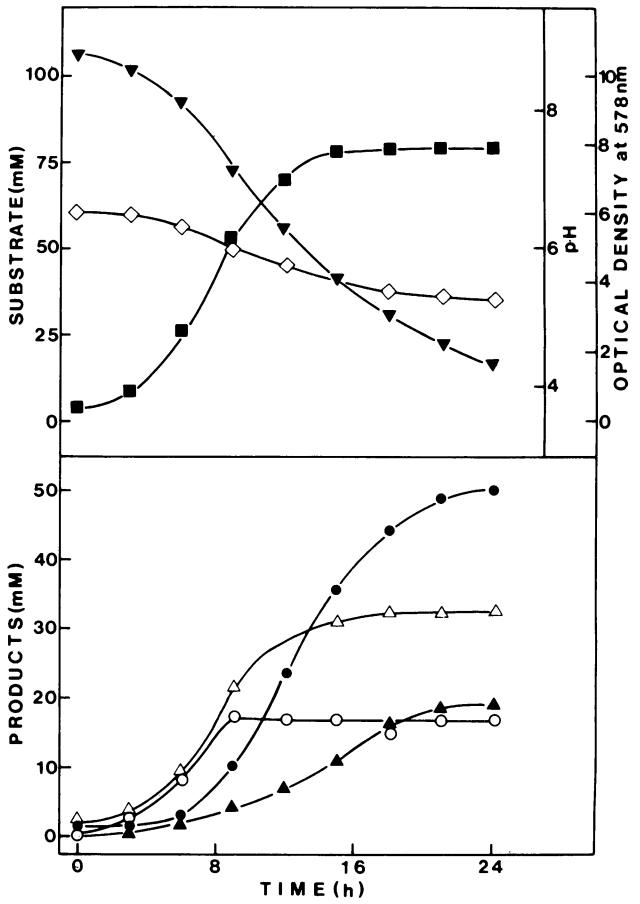

FIG. 1. Growth and product formation of strain MG1 on phosphate-carbonate medium containing 2% (wt/vol) glucose. The experiment was carried out in a Biostat M fermentor (Braun) containing 1 liter of medium. The inoculum was 50 ml of a culture that had been grown in the same medium. Samples of 10 ml were withdrawn and analyzed as described in the text. Symbols: ■, optical density at 578 nm; ◇, pH value; ▼, glucose; ▲, ethanol; △, acetate; ●, butanol; ○, butyrate.

The course of product formation and other growth parameters of strain MG1 are depicted in Fig. 1. Butyrate and acetate were produced, and the pH dropped from 6.5 to 5.2. The formation of *n*-butanol started after a short lag and continued for some time after growth ceased. That *n*-butanol was formed during growth of the organism is apparent from Fig. 2. By using a medium with 6% (wt/vol) glucose and keeping the pH constant at 6.6, *n*-butanol was produced as long as the cells grew. Thereafter, only the formation of ethanol and gaseous products continued.

Taxonomic classification of strain MG1. The new isolate was compared with *C. acetobutylicum*, *C. beijerinckii*, and

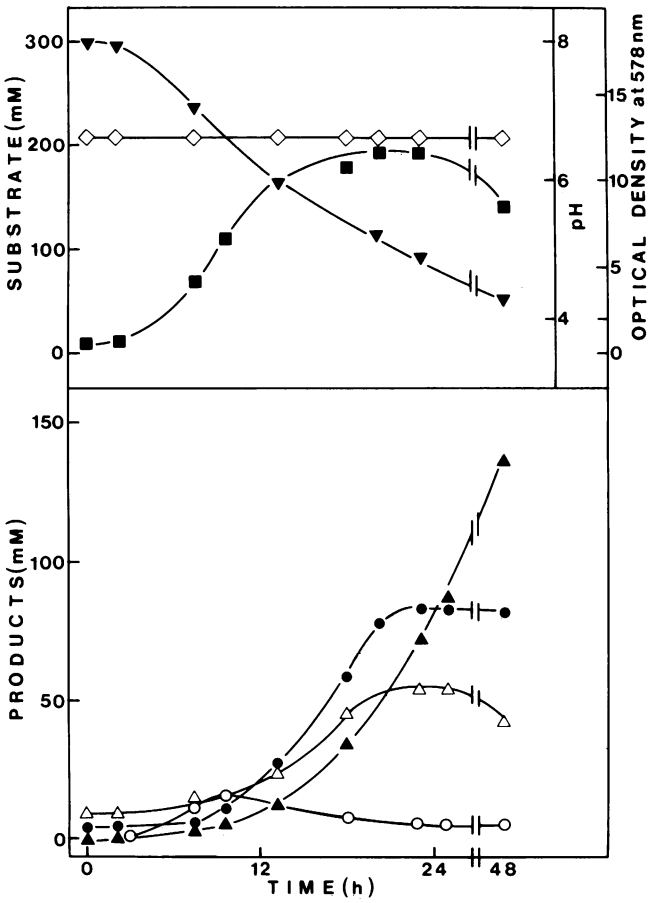

FIG. 2. Growth and product formation of strain MG1 in a medium containing 6% (wt/vol) glucose. The pH was kept at a value of 6.6 throughout the experiment. The experiment was carried out as described in the legend to Fig. 1. Symbols: ■, optical density at 578 nm; ◇, pH value; ▼, glucose; ▲, ethanol; △, acetate; ●, butanol; ○, butyrate.

C. aurantibutyricum in regard to a number of taxonomically relevant properties (Table 3).

Differences were encountered with respect to the optimal growth temperature, the G+C content, indole formation, hydrogen sulfide formation from organic sulfur compounds, utilization of milk, and the form and position of spores. This indicated that the new isolate could not be assigned to one of these species.

Because of the terminal position of the spores in strain MG1 and some correspondence in other properties, MG1 was compared with strains of *C. cochlearium*, *C. lentoputrescens*, and "*C. tetanomorphum*" which had been studied recently (17). On the basis of DNA-DNA homology studies, 11 strains of the above species were subdivided into two groups. One group (seven strains) was designated *C. cochlearium* and the remaining four strains were designated "*C. tetanomorphum*" (Table 1).

In Table 4, the results of experiments are summarized in which strain MG1 was compared with some strains of the *C. cochlearium* and "*C. tetanomorphum*" groups. DNA-DNA hybridization of strain MG1 with strain DSM 665 ("*C. tetanomorphum*") showed 98 to 100% homology.

The strains of the two groups produced only traces of ethanol and *n*-butanol when they were grown on glutamate.

TABLE 3. Comparison of strain MG1 with three *n*-butanol-producing clostridial species[a]

Parameter	*C. aceto-butylicum*	*C. beije-rinckii*[b]	*C. auranti-butyricum*[b]	Strain MG1
Optimal growth temperature (°C)	37	30	30	46
G+C content (mol%)	27	27	27	25
Indole production	−	−	−	+
Lecithinase activity	−	−	−	+
Digestion of milk	+	+	+	−
Hydrolysis of gelatin	+/−	+/−	+	−
Nitrate reduction	−	−	+	−
H_2S production	−	−	−	+
Hemolysis	−	−	−	−
Motility	+	+	+	+
Form of spores	O	O	O	R
Location of spores	ST	ST	ST	T
Fermentation of:				
Glucose, fructose, maltose, mannose, cellobiose, xylose	+	+	+	+
Lactose, starch	+	+	+	−
Ribose, sorbitol	−	−	−	+

[a] Symbols and abbreviations: −, no growth; +, distinct growth (optical density ≥ 1); +/−, growth only with a few strains; O, oval; R, round; ST, subterminal; T, terminal.

[b] Data were taken from Holdemann et al. (12) except for the G+C content (mol%).

Approximately 1.1 mol of acetate and 0.4 mol of butyrate were formed per mol of glutamate consumed by all 11 strains of the two groups. With glucose as substrate, only strains belonging to the "*C. tetanomorphum*" group showed good growth. They reached optical densities of more than 2.0 after 24 h, whereas these values were in the order of 0.6 to 1.3 for the *C. cochlearium* strains. Correspondingly, the product

TABLE 4. Comparison of strain MG1 with the *C. cochlearium* and "*C. tetanomorphum*" groups of Nakamura et al. (17)[a]

Parameter	*C. cochlearium* group	"*C. tetanomorphum*" group	Strain MG1
Range of DNA-DNA homology (%)	81–100	88–100	98–100 with DSM 665
Fermentation of:			
Glucose	+	+	+
Cellobiose, fructose, inositol, maltose, sorbitol	−	+	+
Liquefaction of gelatin	+/−	−	−
Indole formation	+/−	+/−	+
H_2S production	+	+	+
Hemolysis	+/−	−	−
Products on glutamate (mM)[b]:			
Ethanol	0.9–1.0	1.5–2.4	3.5
Acetate	100.5–101.6	100.5–112.7	104.4
n-Butanol	0.6–1.0	2.0–3.2	5.0
Butyrate	32.0–44.0	39.0–39.5	40.5
Products on glucose (mM)[c]:			
Ethanol	0.6–1.1	27.0–43.3	42.7
Acetate	9.5–22.4	24.2–32.7	23.1
n-Butanol	0.3–2.1	37.4–44.6	47.1
Butyrate	5.2–31.3	4.8–7.2	5.3

[a] Abbreviations as in Table 3.

[b] Growth medium contained 100 mM sodium L-glutamate.

[c] Growth medium contained 100 mM D-glucose.

concentrations were different; the members of the *C. cochlearium* group produced only traces to small amounts of *n*-butanol. This solvent, however, was the major fermentation product of strain MG1 and the "*C. tetanomorphum*" strains.

DISCUSSION

The formation of *n*-butanol by *C. acetobutylicum*, *C. beijerinckii*, and *C. aurantibutyricum* is always accompanied by the formation of acetone or isopropanol (11). Species such as *C. pasteurianum*, *C. sporogenes*, and *C. cadaveris* produce only minor amounts of *n*-butanol, and acetone and isopropanol are not detectable (11). Strain MG1, isolated in the course of our attempts to obtain new *C. acetobutylicum* strains, differed from all these species in that it did not form acetone or isopropanol but produced *n*-butanol as a major fermentation product from glucose or other sugars. At a constant pH of 6.6, *n*-butanol formation was associated with growth, and after termination of growth, only ethanol and CO_2 plus H_2 were formed. When the pH was allowed to decrease to pH 5.2 during growth, some butanol was also formed in the stationary phase. *C. acetobutylicum* produces butanol and acetone in association with growth in continuous culture at a low pH (1, 3, 4). In batch culture, the organisms switch over from acetate-butyrate to butanol-acetone formation only after growth ceases, and the pH increases from below 5 to ca. 5.2 (7). As reported for *C. berjerinckii* (10), strain MG1 did not require a low pH for the onset of *n*-butanol formation, and the solvent was formed during exponential growth. The regulation of butanol formation in strain MG1 must, therefore, be different from the one in *C. acetobutylicum*. Whereas morphological changes of *C. acetobutylicum* cells in the direction of spore formation seem to occur before the shift to solvent formation (14), such changes and the formation of spores have not been observed with strain MG1 under the conditions of butanol production. In addition, it has been demonstrated that some enzymes specifically involved in acetone and butanol formation are not present or are only present in low amounts in *C. acetobutylicum* cells from the exponential-growth phase (2). For strain MG1, it can be assumed that these enzymes are synthesized constitutively.

On the basis of the DNA-DNA homology of 98 to 100% between strain MG1 and the *Clostridium* sp. strain DSM 665 (formerly "*C. tetanomorphum*"), the new isolate should be assigned to the "*C. tetanomorphum*" group of Nakamura et al. (17). In the 8th edition of *Bergey's Manual* (6) as well as in the *Approved Lists of Bacterial Names* (18), "*C. tetanomorphum*" is no longer recognized as a species. The reason for this is the results of Takahashi et al. (19), which demonstrated that this species consisted of two distinct groups of strains. Nakamura et al. (17) made an attempt to reclassify these strains and strains of *C. cochlearium* and *C. lentoputrescens*. They suggested that three strains that are distinct from the above species form a new species for which the name "*C. tetanomorphum*" should be retained. This conclusion is fully supported by the data presented here, and additional distinctive properties are reported. Only the "*C. tetanomorphum*" strains show good growth on D-glucose and completely degrade 2% (wt/vol) D-glucose; only these strains produce *n*-butanol as a main fermentation product from D-glucose.

ACKNOWLEDGMENTS

This work was supported by grants from the Bundesministerium für Forschung und Technologie and the Max-Buchner-Stiftung.

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