Clostridium tetanomorphum sp. nov., nom. rev.

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A total of 46 glutamate-degrading strains of the genus *Clostridium*, including 36 new isolates, were examined for deoxyribonucleic acid (DNA)-DNA homology, protein patterns produced by polyacrylamide gel electrophoresis, and physiological characteristics in order to determine the differences between *Clostridium cochlearium* and "*Clostridium tetanomorphum*." Thirty-four newly isolated strains could be assigned to the "*C. tetanomorphum*" group; two strains were classified as nontoxigenic *Clostridium tetani* strains. The levels of DNA-DNA homology between *C. cochlearium* and "*C. tetanomorphum*" were only 10 to 19%, and the protein patterns showed that there were great differences between both groups and other glutamate-degrading species. Biochemical tests supported these results. In contrast to *C. cochlearium*, all "*C. tetanomorphum*" strains fermented carbohydrates (at least glucose and maltose); they also produced lipase and degraded ethanolamine. Moreover, *C. cochlearium*, like other glutamate-degrading species, needed a higher concentration of yeast extract than "*C. tetanomorphum*" when it was grown on glutamate. On the basis of our results we propose *C. tetanomorphum* sp. nov., nom. rev. The type strain is strain NCTC 543 (= DSM 4474).

Formerly, the classical glutamate-fermenting clostridial species included *Clostridium cochlearium* (Douglas, Fleming, and Colebrook 1919) Bergey, Harrison, Breed, Hammer, and Huntoon 1923; *Clostridium tetanomorphum*" (Bullock, Bulloch, Douglas, Henry, McIntosh, O'Brien, Robertson, and Wolf 1919) Bergey et al. 1923; *Clostridium tetani* (Flügge 1886) Bergey et al. 1923; and *Clostridium lentoputrescens* Hartsell and Rettger 1934. Since Bergey's *Manual of Determinative Bacteriology*, 1st ed. (4), and up to Bergey's *Manual*, 7th ed. (5), "*C. tetanomorphum*" was recognized as a species, but "*C. tetanomorphum*" was not included in Bergey's *Manual*, 8th ed. (34), in Bergey's *Manual of Systematic Bacteriology*, vol. 2 (9), or on the Approved Lists of Bacterial Names (32). In 1979 Nakamura et al. (29) showed that the type strains of *C. cochlearium* and *C. lentoputrescens* had a high level of deoxyribonucleic acid (DNA)-DNA homology. *C. cochlearium*, the earlier name, was retained.

Because of the taxonomic studies of Nakamura et al. (29) on *C. tetani* and related species, further investigation of the taxonomic status of "*C. tetanomorphum*" seemed to be necessary. On the basis of DNA-DNA homology studies, Nakamura et al. divided 24 strains into three groups. Three strains of the previously described species "*C. tetanomorphum*" were neither *C. tetani* nor *C. cochlearium* strains (29).

Another clue which made reevaluation of the taxonomic status of the "*C. tetanomorphum*" strains desirable came from physiological studies. It was found that these strains, but not *C. cochlearium* strains, produce considerable amounts of butanol (17, 29).

MATERIALS AND METHODS

Bacteria. Most of the strains included in this study are listed in Table 1; we also included *Clostridium sporosphaeroides* DSM 1294^T (T = type strain), *Clostridium subterminale* DSM 2636^T, and *Clostridium symbiosum* DSM 934^T. For strains which were obtained from the Deutsche

Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Braunschweig, Federal Republic of Germany, we used DSM designations, and for strains which were obtained from the National Collection of Type Cultures (NCTC), London, United Kingdom, we used NCTC designations. "*C. tetanomorphum*" MG1 was isolated and characterized by M. Gottwald (17). Strains E4222, 34946A, L97, and E4539 and the *C. tetani* strains were obtained from human clinical specimens and were isolated by one of us (G.S.). All other strains were isolated from soil and mud samples by using glutamate as the substrate and the procedure described by Barker (3) and Gottschalk et al. (16). The samples were pasteurized at 80, 90, or 100°C or were treated with ethanol (21) in order to isolate *Clostridium* species. The isolates were tested for toxigenicity, and none was positive.

Stock cultures of the strains were maintained in Reinforced Clostridial Medium (Oxoid Ltd., London, United Kingdom) or chopped meat medium (18) by using the anaerobe culture techniques of Hungate (19).

Biochemical tests. Indole production, gelatin hydrolysis, production of lipase, casein digestion, and catalase production were studied by using the methods of Holdeman et al. (18) and the methods recommended in the *Manual of Methods for General Bacteriology* (33).

Lipolytic activity was also tested by using 0.2% (vol/vol) tributyrin in tributyrin agar base (E. Merck AG, Darmstadt, Federal Republic of Germany). A Hungate tube containing 5 ml of agar medium was inoculated with 1 drop of a culture, and upon incubation a progressive clearing from the top to the bottom of the turbid agar column was recorded as positive lipolysis.

Growth tests were carried out in Barker medium (2) containing 1% sodium L-glutamate, 0.03% K₂HPO₄, 0.01% MgSO₄ · 7 H₂O, 0.05 or 0.5% yeast extract, 0.05% L-cysteine hydrochloride, 0.0001% (wt/vol) resazurin, and tap water; this medium was adjusted to pH 7.6 with sodium hydroxide.

Sugar fermentation tests. The sugar fermentation tests were performed as described by Nakamura et al. (29). A total of 17 different sugars were tested in Hungate tubes

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TABLE 1. Bacterial strains studied

Strain(s)	Source	DNA homology group ^a
<i>C. cochlearium</i>		
DSM 666	Hay	
DSM 667	Raw milk	
DSM 1285 ^T (= ATCC 17787 ^T)	Unknown	II
DSM 2153 (= ATCC 17794)	Unknown	II
NCTC 2909 ^b	Saliva	II
" <i>C. tetanomorphum</i> "		
DSM 528 (= ATCC 15920)	Soil	III
DSM 665 (= NCTC 288), NCTC 500, NCTC 543 ^T	Septic wounds	III
MG1	Rhizosphere, bean	III
S1;T2/2	River mud	III
S2;T4/1, S3;T4/2, S4;T4/3, S5;T5/2, S13;Ta/2, 1;T1/1, 2;T1/2, 3;T2/1, 4;T5/1, 23;TA/1, 24;TB/1	River mud	
5;T8/2	Garden soil	III
S7;T7/2b, S8;T7/2c, S12;Ta/1, 6;T7/2a, 9;T7/2d, 6;T8/3, 9;T13/1, 10;T13/4, 11;T13/5, 12;T14/1, 13;T14/4, 14;T15/1, 15;T15/4, 16;T15/2	Garden soil	
10;T10/3, 7;T10/2	Soil, grassland	
11;T12/2b, 8;T12/2	Dung hill	
L97, E4539	Clinical	
22;T21/2	Soil, Pakistan	
<i>C. tetani</i>		
Toxic strains Fö and L27	Clinical	
Nontoxic strains 34946A and E4222	Clinical	I
<i>C. malenominatum</i> DSM 1127 ^T	Unknown	
<i>C. limosum</i> DSM 1400 ^T	Marine mud	

^a Homology groups to which the strains were assigned by Gottwald et al. (17) or Nakamura et al. (29) or in our own studies (see Table 3).

^b Received as "*C. tetanomorphum*" from the National Collection of Type Cultures.

containing 10 ml of P medium (2.0% Proteose peptone, 0.5% NaCl, 0.1% agar, 0.05% L-cysteine hydrochloride, 0.0001% [wt/vol] resazurin, pH 7.2). Sugar solutions (25%, wt/vol) were added to the tubes to give final concentrations of 1% (wt/vol), except for salicin (final concentration, 0.5%). Each tube was inoculated with 0.25 ml of a 1-day-old culture in Reinforced Clostridial Medium. The pH values and the optical densities at 600 nm were measured at the beginning, after 3 days, and after 1 week. The pH measurements were made with a microelectrode in 0.5-ml samples removed anaerobically from the culture tubes. Inoculated P medium without sugar and sterile P medium containing sugar also were incubated and served as controls.

Analysis of fermentation products. Products such as *n*-butanol, ethanol, butyrate, and acetate were determined by gas chromatography as described by Gottwald et al. (17).

Amine fermentation test. Utilization of betaine, choline, and ethanolamine was tested in PY medium (18) supplemented with 50 mM amine. After incubation for 3 days, the culture pH and the optical density at 600 nm were measured and compared with the values for amine-free controls. Utilization of amine was confirmed by thin-layer chromatographic analysis, using the method of Möller et al. (27).

Determination of DNA base composition and DNA-DNA homology. All strains were cultivated anaerobically at 37°C in flasks containing 10 liters of PY medium (18), which contained the following components: peptone, 0.5%; Trypticase peptone, 0.5%; yeast extract, 1%; resazurin, 0.0001%; L-cysteine hydrochloride, 0.05% (wt/vol); and salt solution, 4% (vol/vol). The cells were harvested at the early exponential phase of growth by using a continuous-flow centrifuge at 35,000 × *g*, washed with saline ethylenediaminetetraacetate (0.15 M NaCl, 0.001 M ethylenediaminetetraacetate, pH 8), resuspended, and stored at -20°C.

At 1 h before harvest, glycine was added to the medium to give a concentration of 0.4% (wt/vol). This was necessary to

achieve lysis of the cells by lysozyme and sodium dodecyl sulfate (SDS).

DNA was isolated by using the method of Marmur (25). The purity of the DNA was tested by spectrophotometry (ratio of optical density at 260 nm to optical density at 230 nm to optical density at 280 nm, 1.0:0.450:0.515) with a spectrophotometer (Perkin-Elmer, Überlingen, Federal Republic of Germany).

The guanine-plus-cytosine (G+C) contents of the DNAs were determined by thermal denaturation as described previously (24, 26). DNA of *Escherichia coli* K-12 DSM 498 (51.7 mol% G+C) was used as the reference.

Levels of DNA-DNA homology were determined by measuring the renaturation rates of the denatured DNAs at the optimal renaturation temperatures as recommended by De Ley et al. (12) and Huss et al. (20).

A model 250 spectrophotometer (Gilford Instrument Laboratories, Inc., Düsseldorf, Federal Republic of Germany) equipped with a model 2527 thermoprogrammer, a model 1100 recorder (Gilford), and hermetically sealed thermocuvettes was used.

Gel electrophoresis of soluble proteins. The gel electrophoresis procedures of Moore et al. (28) and Cato et al. (10) were modified as described below.

The strains were grown in Hungate tubes containing 10 ml of PY medium and were harvested after 16 to 18 h in the late exponential growth phase by centrifugation at 8,000 × *g* for 15 min. The cells were washed and were suspended in 0.3 to 0.5 ml of 0.1 M tris(hydroxymethyl)aminomethane chloride buffer containing 1% (wt/vol) MgCl₂ (pH 7.4). Each cell suspension was passed twice through a French pressure cell at 8,000 Pa and was centrifuged at 15,000 × *g* for 5 min to remove cell debris. The protein concentrations of the supernatants were determined by the method of Lowry et al. (23); 100 µg of protein from each strain was needed as a sample for gel electrophoresis.

TABLE 2. G+C contents of DNAs from selected strains

Strain	Thermal denaturation temp (°C)	G+C content (mol%)
<i>C. cochlearium</i>		
DSM 667	79.45	25
DSM 2153	79.5	25
NCTC 2909	80.35	27
" <i>C. tetanomorphum</i> "		
DSM 528	80.16	27
DSM 665	80.6	28
NCTC 500	79.7	25
NCTC 543 ^T	80.55	28
MG1	81.0	29
<i>C. tetani</i> (nontoxic)		
34946A	80.0	26
E4222	79.9	26
<i>C. malenominatum</i> DSM 1127 ^T	81.25	29

Cells of *C. tetani* were broken in another way in order to avoid any intoxication. They were grown in the common manner, but they were washed and suspended in 0.1 M tris(hydroxymethyl)aminomethane chloride buffer containing MgCl₂ and an additional 2% (wt/vol) SDS. Then the cell suspensions were heated at 80°C for 10 min. The cell extracts were prepared with lysozyme instead of French pressing; 2 mg of lysozyme was added to 500 µl of a cell suspension, which was incubated at 37°C for 30 min.

For gel electrophoresis 100 µg of protein in 10 µl of 10 mM sodium phosphate buffer (pH 7.0) was incubated with 3% (vol/vol) 2-mercaptoethanol and 2% (wt/vol) SDS for 5 min at 100°C.

The denatured proteins were mixed with 5 µl of mercaptoethanol, 5 µl of bromophenol blue (0.05% [wt/vol] in 10 mM sodium phosphate buffer, pH 7.0), and some crystals of sucrose and were applied to the gels. SDS-polyacrylamide gel electrophoresis by the method of Laemmli (22) was performed in a vertical tank apparatus. The lower gel contained 11.5% acrylamide, and the sample gel contained 4.5% acrylamide. The running buffer contained 3.0 g of tris(hydroxymethyl)aminomethane base, 24.4 g of glycine, and 1.0 g of SDS in 1,000 ml of water. Each electrophoresis run included a mixture of defined proteins (albumin, chymotrypsinogen, and cytochrome *c*; Boehringer GmbH, Mannheim, Federal Republic of Germany). Electrophoresis was performed at 4°C and a constant voltage of 150 V with an initial current of about 33 mA. Electrophoresis was stopped after 5 to 7 h, when the tracking dye reached the bottom of the gel. The gels were removed from the plates and were placed overnight in a fixing bath containing 2.25 ml of methanol, 46 ml of glacial acid, and 2.29 ml of water (30). They were stained with Coomassie blue for 5 days and destained in *o*-phosphoric acid (2%, vol/vol), as described by Neuhoft et al. (30).

RESULTS

G+C content and DNA-DNA homology. DNA base compositions were determined for 11 strains (Table 2). The G+C values did not allow group separation.

Representative strains (strains DSM 667 and DSM 2153 of *C. cochlearium* and strains NCTC 500, NCTC 543^T, and MG1 of the "*C. tetanomorphum*" group) were selected to determine the levels of DNA-DNA relatedness between these groups and other strains of unclear taxonomic position

TABLE 3. Levels of DNA-DNA relatedness between representative strains

Source of reference DNA	% Relatedness to:				
	" <i>C. tetanomorphum</i> " strains			<i>C. tetani</i> strains (nontoxic)	
	DSM 528	S1;T2/2	5;T8/2	34946A	E4222
<i>C. cochlearium</i>					
DSM 667				59	
DSM 2153	19	10		51	51
" <i>C. tetanomorphum</i> "					
NCTC 500					49
NCTC 543 ^T	82			34	38
MG1		93	82		
S1;T2/2			100	13	
<i>C. tetani</i> 34946A (nontoxic)					98
<i>C. malenominatum</i> DSM 1127 ^T					22

(Table 3). The position of strain DSM 528, which was added to the "*C. tetanomorphum*" group because of physiological characteristics (17), was confirmed by a DNA-DNA relatedness value of 82% with "*C. tetanomorphum*" DSM 543^T.

Two new isolates, strains S1;T2/2 and 5;T8/2, exhibited high levels of DNA-DNA relatedness to strain MG1 and clearly belonged to the "*C. tetanomorphum*" group.

Strains 34946A and E4222 did not belong to either the "*C. tetanomorphum*" group or *C. cochlearium* based on the low levels of DNA-DNA relatedness to representatives of these groups. They also were not closely related to the glutamate-fermenting species *Clostridium malenominatum*, as shown by a value of 22%.

Protein patterns obtained by gel electrophoresis. To get comparable protein patterns for all strains, the strains were cultivated in the same medium (PY medium) and were harvested at the end of the exponential growth phase. More than 30 protein bands could be distinguished on the individual gels.

The differences between type and reference strains of *C. cochlearium* and strains of the "*C. tetanomorphum*" group are shown in Fig. 1. Four bands were typical of *C. cochlearium*, whereas seven bands were typical of "*C. tetanomorphum*." The patterns for strains 34946A and E4222 differed from the patterns for both groups, confirming the DNA-DNA relatedness results. The protein patterns of these strains were very similar to those of *C. tetani* (Fig. 2). Results of physiological tests also indicated that both of these nontoxic strains belong to the species *C. tetani*.

The protein patterns of all 34 new isolates were determined and were identical to those of "*C. tetanomorphum*" DSM 665, NCTC 500, and NCTC 543^T and different from the pattern of *C. cochlearium* DSM 667. Some comparisons with "*C. tetanomorphum*" NCTC 543 are shown in Fig. 3. The similarity among the patterns is apparent. All 34 strains may be classified as "*C. tetanomorphum*"; this was confirmed by the high levels of DNA-DNA relatedness between strain 5;T8/2 of this group and "*C. tetanomorphum*" MG1, as well as by the levels of DNA-DNA relatedness for strain S1;T2/2 with strains of "*C. tetanomorphum*," *C. cochlearium*, and *C. tetani* (Table 3).

To determine the limits between "*C. tetanomorphum*" and other glutamate-degrading species, the protein patterns of "*C. tetanomorphum*," *C. cochlearium*, and *C. tetani* were compared with those of type strains *Clostridium limo-*

FIG. 1. Polyacrylamide gel electrophoretic protein patterns of *C. cochlearium* DSM 666, DSM 667, DSM 1285^T, DSM 2153, and NCTC 2909, "*C. tetanomorphum*" DSM 528, DSM 665, NCTC 500, NCTC 543^T, and MG1, and strains E4222 and 34946A.

sum (strain DSM 1400) and *C. malenominatum* (strain DSM 1127). The differences among *C. limosum*, *C. malenominatum*, "*C. tetanomorphum*," *C. cochlearium*, and *C. tetani* are shown in Fig. 4.

Biochemical properties. The results of protein gel electrophoresis and of DNA-DNA relatedness studies of some selected strains allowed exact differentiation between the glutamate-degrading strains. In a search for corresponding biochemical properties, degradation of 17 sugars was tested by measuring the final pH values and the optical densities at 600 nm. The test was read as positive when the pH value of a culture that was incubated for 1 week dropped more than 0.5 U or the optical density at 600 nm of such a culture reached a value higher than 1.0 U; both measurements were determined in relation to controls. Determination of optical density was necessary in cases in which the fermentation of sugars, such as cellobiose, fructose, and salicin, did not lead to a remarkable decrease in the pH but did lead to an increase in growth.

C. malenominatum DSM 1127^T and *C. cochlearium* DSM 666, DSM 667, DSM 1285^T, DSM 2153, and NCTC 2909 did not ferment any of the 17 carbohydrates tested, whereas all of the strains belonging to the "*C. tetanomorphum*" group fermented at least four sugars. Glucose and maltose were fermented by all of the "*C. tetanomorphum*" strains; xylose, inositol, ribose, sorbitol, cellobiose, salicin, and fructose were fermented by 95, 87, 79, 55, 42, 15, and 6% of the strains, respectively. "*C. tetanomorphum*" DSM 528, DSM 665, NCTC 500, NCTC 543^T, and MG1 were not able to utilize adonitol, arabinose, galactose, lactose, mannose, rhamnose, saccharose, or sorbose.

The only sugar which strain E4222 fermented was inositol, which corresponded to the data of Nakamura et al. (29) for *C. tetani*.

FIG. 2. Polyacrylamide gel electrophoretic protein patterns showing the similarities between strains of *C. tetani* and strains E4222 and 34946A. The prominent band at the bottom of the gel resulted from lysozyme, which was used for breaking the cells.

FIG. 3. Polyacrylamide gel electrophoretic protein patterns showing the similarities between 16 newly isolated strains and "*C. tetanomorphum*" NCTC 543^T.

FIG. 4. Polyacrylamide gel electrophoretic protein patterns of the reference strains of the five glutamate-degrading species *C. cochlearium*, "*C. tetanomorphum*," *C. tetani*, *C. limosum*, and *C. malenominatum*.

C. cochlearium, *C. limosum*, and *C. malenominatum* did not produce lipase, whereas all of the strains belonging to the "*C. tetanomorphum*" group, including the new isolates, and *C. tetani* were lipase positive (Table 4). The lipolytic activity of these strains varied obviously with the quality of egg yolk used, but lipolytic activity was a constant property, when organisms were tested with tributyrin.

No strain of *C. cochlearium* nor the type strain of *C. malenominatum* degraded ethanolamine, but *C. limosum*, *C. tetani*, and all of the "*C. tetanomorphum*" strains metabolized this amine. Therefore, the production of lipase (tributyrin) and the degradation of ethanolamine were suitable characteristics for differentiation between *C. cochlearium* and "*C. tetanomorphum*."

Choline was degraded by *C. limosum* and was weakly degraded by three of the four *C. tetani* strains tested.

Three strains of *C. cochlearium*, including the type strain, all four strains of *C. tetani*, and *C. limosum* degraded gelatin when the organisms were tested in 12% gelatin medium, although most of the strains produced only a weak reaction. However, all eight strains were positive on 0.4% gelatin agar. None of the "*C. tetanomorphum*" strains, including the freshly isolated strains, hydrolyzed gelatin when they were tested by both methods. However, this property is of no differential value because it varied for strains of *C. cochlearium*.

The production of indole was variable in cultures of all species except *C. limosum*. Therefore, this biochemical property could not be used to differentiate the glutamate-degrading clostridia.

When the growth of all strains was tested in glutamate medium supplemented with two different concentrations of yeast extract, remarkable differences were recorded. Only the strains of "*C. tetanomorphum*" grew well in the presence of 0.05% yeast extract, whereas *C. cochlearium*, *C.*

TABLE 4. Differential features of glutamate-fermenting species

Characteristic	<i>C. cochlearium</i>	" <i>C. tetanomorphum</i> "	<i>C. tetani</i>	<i>C. malenominatum</i>	<i>C. limosum</i>
Degradation of:					
Ethanolamine	0/5 ^a	39/39	4/4	0/1	1/1
Choline	0/5	0/39	3/4	0/1	1/1
Hydrolysis of gelatin	3/5	0/39	4/4	0/1 ^b	1/1
Production of:					
Indole	3/5	31/39	3/4	1/1	0/1
Lipase (tributyryl)	0/5	37/39	4/4	0/1	0/1
Lecithinase	0/5	0/39	0/4	0/1	1/1
Fermentation of:					
Cellobiose	0/5	16/38	0/2	0/1	0/1 ^b
Fructose	0/5	6/38	0/2	0/1	0/1 ^b
Glucose	0/5	38/38	0/2	0/1	0/1 ^b
Inositol	0/5	35/38	1/2	0/1	0/1 ^b
Maltose	0/5	38/38	0/2	0/1	0/1 ^b
Ribose	0/5	30/38	0/2	0/1	0/1 ^b
Salicin	0/5	15/38	0/2	0/1	0/1 ^b
Sorbitol	0/5	21/38	0/2	0/1	0/1 ^b
Xylose	0/5	36/38	0/2	0/1	0/1 ^b
Adonitol, arabinose, galactose, lactose, mannose, rhamnose, saccharose, sorbose	0/5	0/5	0/2	0/1	0/1 ^b
Growth in Barker medium containing 0.05% yeast extract ^c	0.270–0.600	1.120–1.210	0.130–0.180	0.380	0.000

^a Number of strains positive/number of strains tested.^b Data from reference 9.^c Maximum optical density of at 600 nm reached after two transfers.

tetani, *C. malenominatum*, and some other glutamate-fermenting clostridial species needed at least 0.5% yeast extract (Table 5).

DISCUSSION

In 1939 Barker (3) stated that the ability to ferment glutamate was restricted to relatively few species. Based on differences in the utilization of glucose, he assigned the strains which he isolated to one of two species, *C. cochlearium* (glucose negative) or "*C. tetanomorphum*" (glucose

positive), both of which originally were described by Bulloch et al. in 1919 (8). These two species were placed in the genus *Clostridium* in 1923 by Bergey et al. (4). Subsequently, isolates of *C. tetani* and *C. lentoputrescens* were also recognized as glutamate-degrading clostridia (6, 11, 14).

In 1967 Takahashi et al. (35) reported that two groups of distinctly different strains had been labeled "*C. tetanomorphum*." By using DNA-DNA homology studies, Nakamura et al. (29) demonstrated that one group consisted of strains of *C. cochlearium*. The same authors believed that the other group of strains belonged to "*C. tetanomorphum*." The level of intergroup homology was 9 to 41%. Unfortunately,

TABLE 5. Growth of glutamate-fermenting strains in 1% glutamate–mineral medium supplemented with 0.05 or 0.5% yeast extract^a

Strain(s)	Optical density at 600 nm in:	
	Medium containing 0.05% yeast extract	Medium containing 0.5% yeast extract
<i>C. cochlearium</i> DSM 666, DSM 667, DSM 1285 ^T , DSM 2153, NCTC 2909	0.270–0.600	1.470–1.780
" <i>C. tetanomorphum</i> "		
DSM 528, DSM 665, NCTC 500, NCTC 543 ^T , MG1	1.120–1.210	1.360–1.880
S1;T2/2, S2;T4/1, S3;T4/2, S4;T4/3, S5;T5/2, S7;T7/2b, S8;T7/2c, S12;Ta/1, S13;Ta/2, 6;T7/2a, 9;T7/2d, 10;T10/3, 11;T12/2b	0.950–1.600	1.300–>2.000
L97, E 4539, 1;T1/1, 2;T1/2, 3;T2/1, 4;T5/1, 5;T8/2, 6;T8/3, 8;T12/2, 9;T13/1, 10;T13/4, 11;T13/5, 12;T14/1, 13;T14/4, 14;T15/1, 15;T15/4, 16;T15/2, 22;T21/2, 23;TA/1, 24;TB/1		
<i>C. tetani</i>		
Toxic strains Fö and L27	0.140–0.180	0.900–1.300
Nontoxic strains 34946A and E4222	0.130–0.170	1.300–1.450
<i>C. malenominatum</i> DSM 1127 ^T	0.380	1.300
<i>C. limosum</i> DSM 1400 ^T	0.000	0.100
<i>C. sporosphaeroides</i> DSM 1294 ^T	0.000	0.690
<i>C. subterminale</i> DSM 2636 ^T	0.000	0.400
<i>C. symbiosum</i> DSM 934 ^T	0.170	0.010

^a Maximum optical densities of cultures were measured directly in Hungate culture tubes by using a spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). Measurements were made after a second transfer into the medium indicated.

the name "*C. tetanomorphum*" was not included on the Approved Lists of Bacterial Names (32).

In our attempts to isolate new strains of glutamate-degrading clostridia, we obtained almost exclusively strains of the "*C. tetanomorphum*" group. A total of 34 newly isolated strains could be assigned to "*C. tetanomorphum*" and were different from *C. cochlearium* and toxigenic or nontoxigenic *C. tetani* strains.

The reinstatement of the species "*C. tetanomorphum*" and its separation from *C. cochlearium* seem to be justified based on the results of DNA-DNA studies (Table 3).

In *Bergey's Manual of Systematic Bacteriology*, vol. 2, Cato et al. (9) gave as a reason for the omission of the species "*C. tetanomorphum*" the fact that neither strain ATCC 15920 (= DSM 528) nor strain ATCC 8606 had the characteristics originally described for the species. However, in our studies strain DSM 528 exhibited levels of DNA-DNA homology to *C. cochlearium* DSM 2153 and "*C. tetanomorphum*" NCTC 543^T of 19 and 82%, respectively (Table 3). Physiologically, strain DSM 528 corresponds (based on our results) to the description of "*C. tetanomorphum*" given previously (1, 4, 16, 17). It differs from *C. cochlearium* in that it is able to ferment glucose and some other carbohydrates; *n*-butanol is the main fermentation product from D-glucose.

Unfortunately, the results of Nakamura et al. (29) differ from our results with respect to sugar fermentation. Nakamura et al. (29) found an ability to ferment sugars even in *C. cochlearium* strains.

Table 4 summarizes the physiological differences among the five described species that use the methylaspartate pathway for glutamate breakdown (6, 7). According to the identification key for clostridia given in *Bergey's Manual* (9), "*C. tetanomorphum*" should be separated from the other four species on the basis of the first question; it belongs in group I.A (production of acid from glucose). Here, indole-negative strains lead to *Clostridium pasteurianum*, while indole-positive strains lead to *Clostridium indolis*, *Clostridium sphenoides*, and *Clostridium clostridioforme*. "*C. tetanomorphum*" differs from these species in several characteristics (e.g., lipase formation, sugar utilization pattern, and fermentation of glutamate and histidine). Furthermore, the latter species are unable to utilize ethanolamine (27). Therefore, the identification key in its present form cannot be applied to the "*C. tetanomorphum*" strains.

The differentiation of the other four glutamate degraders, except *C. limosum*, which produces lecithinase but no butyrate as a metabolic product, appears to be difficult, particularly when nontoxigenic strains of *C. tetani* are considered. Our results for these species essentially agree with the characteristics given by Holdeman et al. in the *Anaerobe Laboratory Manual* (18) and by Cato et al. in *Bergey's Manual* (9). However, our four *C. tetani* strains were lipase positive on tributyrin agar. Positive lipolysis on Tween agar has also been reported by Gonzales and Sierra (15).

Differentiation of the species *C. cochlearium* and *C. malenominatum* seems to be difficult because of the production of indole, which is regarded as typical for *C. malenominatum*. We found that some strains of *C. cochlearium* also produce indole, although we used the method described in the *Manual of Methods for General Bacteriology* (33), which is regarded as less sensitive. Cato et al. (10) and Nakamura et al. (29) also stated that indole production is a variable characteristic among strains of *C. cochlearium*.

Our results concerning the liquefaction of gelatin are similar to those of Nakamura et al. (29). We used 12 and

0.4% (wt/vol) gelatin and the method of Holdeman et al. (18) and found that all *C. tetani* strains and three strains of *C. cochlearium* were able to attack gelatin.

Cellular protein patterns obtained by gel electrophoresis are useful for differentiation of the glutamate-degrading species. Cato et al. (10) stated that the degree of homogeneity of protein patterns among strains varied for different species and bore a strong relationship to the degree of DNA-DNA homology. Strains with more than 80% DNA-DNA homology usually produced identical patterns. High levels of similarity among the 39 "*C. tetanomorphum*" strains tested are evident from our results, and there are significant differences in protein patterns between *C. cochlearium* and "*C. tetanomorphum*." The differences are greater than the differences between two previously recognized species, *C. cochlearium* and *C. tetani*.

On the basis of our results we propose establishing a new species, reviving the name *C. tetanomorphum*.

***Clostridium tetanomorphum* sp. nov., nom. rev.** *Clostridium tetanomorphum* (te.ta.no.mor'phum. Gr. n. *tetanus*, tetanus; Gr. n. *morphe*, shape; M.L. adj. *tetano-morphum*, [*Clostridium*] *tetani* shaped). The description below is based on our own data, on data in *Bergey's Manual* (4, 5), and on the data of Nakamura et al. (29) and some other authors.

Cells are slender rods (0.7 to 1.0 by 3.0 to 10.0 μ m) with rounded to tapered ends, are occasionally slightly curved, occur singly or in pairs, and are motile by peritrichous flagella. Spores are spherical or elliptical, terminal to subterminal, swelling the cells. Gram positive.

Surface colonies on PY agar are irregular with lobate margins, 2 to 5 mm in diameter, flat, gray, and semitranslucent with glossy surfaces. There is a tendency to swarm on moist plates. Colonies on PYG agar are white, opaque, and convex, with a mottled internal structure.

Glucose and maltose are fermented; acid and gas are produced. The pH of cultures in glucose media usually is lowered from 6.9 to 5.0 after 5 days. Most strains, including the type strain, also ferment inositol, ribose, and xylose. Fewer strains ferment cellobiose, fructose, salicin, and sorbitol; the type strain utilizes fructose, salicin (weakly), and sorbitol. Growth is strongly stimulated in media containing a fermentable carbohydrate. Arabinose, adonitol, galactose, lactose, mannose, rhamnose, saccharose, and sorbose are not fermented.

Products of glucose fermentation are *n*-butanol, ethanol, acetate, and butyrate.

Glutamate is fermented via the methylaspartate pathway to acetate, butyrate, CO₂, and NH₃ (7).

Histidine is fermented (36). Threonine is degraded to propionate (14); tyrosine is degraded to phenol (14).

Ethanolamine is fermented to acetate, ethanol, and NH₃; during growth the pH is lowered usually by 1 U. Betaine, choline, and creatine are not fermented.

Lecithinase is not produced; lipase is produced on tributyrin; indole production is variable; and gelatin is not hydrolyzed.

Meat, casein, and milk are not digested.

Catalase is not produced.

Nitrate is generally not reduced (29); some strains may be positive for this characteristic (31).

Good growth occurs in glutamate media with a low complex nutritional content (e.g., 0.05% yeast extract).

The optimum temperature for growth is 37°C.

Anaerobic.

Nontoxigenic.

The cell wall peptidoglycan contains *meso*-diaminopimelic acid (37).

The G+C content of the DNA (as determined by the thermal denaturation method) is 25.4 to 28.5 mol%; (27.5 mol% for strain NCTC 543^T).

The type strain is strain NCTC 543 (= DSM 4474), which was isolated as strain 259E^T in 1920 from septic wounds by M. Robertson, Lister Institute, London, United Kingdom.

ACKNOWLEDGMENT

This work was supported by a grant from the Akademie der Wissenschaften zu Göttingen.

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