Taxonomy of *Clostridium tetani* and Related Species

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Clostridium tetani and its related species *C. tetanomorphum*, *C. cochlearium* and *C. lentoputrescens* were examined for DNA–DNA homology and biochemical properties. Two distinctly different groups were included under the name of *C. tetanomorphum*: one was identical with *C. cochlearium* and the name *C. tetanomorphum* was applied to the other group with some amendment of biochemical properties. Comparison of the type strain of *C. lentoputrescens* with wild strains obtained from horse faeces indicated that the name *C. lentoputrescens* should be abolished as a later synonym of *C. cochlearium*. Liquefaction of gelatin and spore shape, which have been used as the important criteria for differentiation of *C. tetani*-related species, were genetically insignificant.

INTRODUCTION

This study has investigated the relationships between *Clostridium tetani*-related species, i.e. *C. tetani* (Flügge) Bergey *et al.* 1923, *C. cochlearium* (Douglas, Fleming & Colebrook) Bergey *et al.* 1923 and *C. lentoputrescens* Hartsell & Rettger 1934. *Bacillus tetanomorphum* and *Bacillus cochlearius* were established in 1919 by Bulloch *et al.* (1919) and the genus name *Clostridium* was applied to them in the first edition of *Bergey's Manual of Determinative Bacteriology* (Bergey *et al.*, 1923). Since then, the name *C. tetanomorphum* has appeared in the *Manual* up to the seventh edition (Breed *et al.*, 1957) but has disappeared from the eighth edition (Smith & Hobbs, 1974). Takahashi *et al.* (1967) have previously reported that two distinctly different groups were included under the name *C. tetanomorphum*. In the present study, tests for DNA–DNA homology, thermostability of DNA duplexes and biochemical properties were used to determine to which group the name of *C. tetanomorphum* should be applied. Furthermore, a possible identity of *C. cochlearium* and *C. lentoputrescens* has been re-investigated in the light of DNA–DNA homology determinations.

METHODS

Strains. These are listed, with the names as received, in Table 1. Strain K-130 was recovered from *C. tetani* HA-47 after heating at 100 °C for 30 min (Nishida *et al.*, 1969). Non-toxicogenic strains M-2, M-6 and M-14 have all the characteristics of *C. tetani* except toxicity.

Preparation of DNA. *Clostridium tetani* HA-47, CN 655, 1110, 1113, 1303, 6114, 6116, PO5 and 007, and *C. tetani*-like strain K-130 were grown at 37 °C in THPYG medium (pH 7.2) containing (% w/v): trypticase (BBL), 0.5; heart infusion broth (Nissui, Tokyo, Japan), 0.5; polipeptone (Daigo, Osaka, Japan), 1.0; yeast extract (Daigo), 1.0; glucose, 1.0; NaCl, 0.5; L-cysteine.HCl, 0.05. *Clostridium tetani* M-20, *C. tetanomorphum* 500 and 543, *C. lentoputrescens* 17794 and *C. tetani*-like strains M-2, M-6 and M-14 were grown in PYG medium (pH 7.2) containing (% w/v): polipeptone (Daigo), 2.0; yeast extract (Daigo), 1.0; glucose, 1.0; NaCl, 0.5; L-cysteine.HCl, 0.05. Penicillin G (50 µg ml⁻¹) was added in the late-exponential phase of growth and cultures were incubated for a further 30 to 45 min at 37 °C. The organisms were then harvested by centrifugation, resuspended in 0.15 M-NaCl/0.01 M-EDTA at pH 8.0 and lysed with 3 % (w/v) sodium dodecyl sulphate (SDS). Bacterial suspensions of strains 543 and 17794 in NaCl/EDTA solution were heated

Table 1. *Source and designation of bacterial strains*

Name (as received)	Strain designation	Source
<i>Clostridium tetani</i>	Havard A-47 (HA-47)	M. Yoneda*
	CN 655	Pasteur Institute (Paris)
	1110, 1113, 1303, 6114, 6116	Stock strains (Sanada & Nishida, 1965)
	PO5, 007, M-20	Stock strains
<i>C. cochlearium</i>	17787	ATCC. Type strain
	montréal	Pasteur Institute (Paris)
<i>C. lentoputrescens</i>	17794	ATCC. Type strain
<i>C. tetanomorphum</i>	1772 C, CIA, A	Pasteur Institute (Paris)
	2909, 288, 500, 543	NCTC
<i>C. tetani</i> -like strains	K-130	Stock strain (Nishida <i>et al.</i> , 1969)
	M-2, M-6, M-14	Stock strains

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at 60 °C for 20 min, before treatment with 1 mg lysozyme ml⁻¹ (Seikagaku, Tokyo, Japan) and 1 mg pronase ml⁻¹ (Kaken, Toyko, Japan) for 10 min at 37 °C and lysis with SDS. *Clostridium cochlearium* 17787 and montréal, and *C. tetanomorphum* 1772 C, CIA, A, 2909 and 288 were grown at 37 °C for 6 to 11 h in PYG medium. The organisms were then harvested by centrifugation, suspended in NaCl/EDTA solution, treated with 1 mg lysozyme ml⁻¹ for 20 min at 37 °C and lysed with 3 % (w/v) SDS. Bacterial suspensions of strains A and 1772 C in NaCl/EDTA solution were heated at 60 °C for 10 min before lysozyme treatment.

For the preparation of labelled DNA from *C. tetani* HA-47 and *C. tetanomorphum* 543, [³H]thymidine (10 µCi; Japan Isotope Association, Tokyo, Japan) was added to 100 ml of the medium used for the isolation of unlabelled DNA of each strain. For the preparation of labelled DNA from *C. cochlearium* 17787, [³H]-thymidine (10 µCi) was added to 100 ml medium containing (% w/v): trypticase (BBL), 3.0; brain heart infusion (Nissan, Tokyo, Japan), 0.3; glucose, 1.0; L-cysteine.HCl, 0.05; mineral salts (Holdeman & Moore, 1975) and vitamins (Cummins & Johnson, 1971). The organisms were incubated, treated and lysed by the procedure used for preparation of unlabelled DNA.

In all cases DNA was extracted from the lysate by the method of Marmur (1961).

Tests for DNA-DNA homology and the thermostability of DNA duplexes. These were performed as described previously (Nakamura *et al.*, 1974).

Sugar fermentation tests. Sugars tested were adonitol, amygdalin, arabinose, cellobiose, dulcitol, aesculin, erythritol, fructose, galactose, glucose, glycerol, glycogen, inositol, inulin, lactose, maltose, mannose, mannitol, melizitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sorbose, starch, sucrose, trehalose and xylose. The basal medium was P medium (pH 7.2) containing (% w/v): proteose peptone no. 2 (Difco), 2.0; NaCl, 0.5; agar, 0.1; L-cysteine.HCl, 0.05. Each sterile sugar was added to 3.5 ml P medium held in a small test tube to give 1 % (w/v), except for glycogen, inulin and salicin which were added at 0.5 % (w/v). Each tube was inoculated with 0.15 ml of overnight culture in liver broth and incubated at 37 °C for 7 d. The pH values of cultures were measured with a pH meter. Fermentation of a sugar was read as positive when the pH difference (ΔpH) between cultures with and without sugar was more than 0.5.

Other biochemical tests. Tests for reduction of nitrate, production of indole, digestion of coagulated albumen, production of H₂S, haemolysis, growth at 0 °C and production of lecithinase were performed according to Nakamura *et al.* (1973). Digestion of casein and milk were examined by the method of Holdeman & Moore (1975). Liquefaction of gelatin was examined by the methods of Nakamura *et al.* (1973) and Holdeman & Moore (1975).

Analysis of fermentation products. Strains tested were incubated for 72 h in PY medium (Holdeman & Moore, 1975). Fatty acids and alcoholic fermentation products were extracted from the cultures and detected by gas chromatography according to Holdeman & Moore (1975), using a Nihon Denshi gas chromatograph model JCG-1100 (Nihon Denshi, Tokyo, Japan).

Spore morphology. A loopful of an overnight culture at 37 °C in liver broth was streaked on PM medium (pH 7.4) containing (% w/v): proteose peptone (Difco), 3.0; meat extract (Daigo), 1.0; NaCl, 0.2; agar, 2.0. The plate was incubated anaerobically under hydrogen at 37 °C for 96 h. Colonies were smeared, and stained by the method of Wirtz (1908) or by the Gram stain.

Isolation of C. tetani-like strains from horse faeces. About 5 g of each horse faeces sample was suspended in 10 ml saline and heated at 60 °C for 10 min. A 1 ml portion of the heated sample was transferred to 10 ml

Table 2. Polynucleotide sequence relationship among *C. tetani* and allied species

Group	DNA source	Labelled DNA source				
		Strain HA-47		Strain 17787		Strain 543
		% Homo- logy	$\Delta T_{m(e)}$ * (°C)	% Homo- logy	$\Delta T_{m(e)}$ * (°C)	% Homo- logy
I	<i>C. tetani</i> HA-47	100	0	64	9.0	16
	<i>C. tetani</i> CN 655	95				
	<i>C. tetani</i> 1113	95				
	<i>C. tetani</i> 6114	94				
	<i>C. tetani</i> -like strain M-14	93	0	46		12
	<i>C. tetani</i> 1110	92		68		
	<i>C. tetani</i> 1303	92				
	<i>C. tetani</i> PO5	92				
	<i>C. tetani</i> M-20	92	0	52		8
	<i>C. tetani</i> 6116	90				
	<i>C. tetani</i> 007	89				
	<i>C. tetani</i> -like strain M-2	89	0.5	38		
	<i>C. tetani</i> -like strain M-6	85	0.5	52	10.0	
	<i>C. cochlearium</i> 17787	56	10.0	100	0	9
II	<i>C. lentoputrescens</i> 17794	44	11.0	100	0.3	9
	<i>C. tetanomorphum</i> 1772 C	51	9.3	96	0	
	<i>C. tetanomorphum</i> 2909	68		91		
	<i>C. tetani</i> -like strain K-130	65		91		
	<i>C. cochlearium</i> montréal	52		85		
	<i>C. tetanomorphum</i> A	61	10.0	83	0.5	
	<i>C. tetanomorphum</i> CIA	64		81		
	<i>C. tetanomorphum</i> 543	40	14.5	28	13.3	100
III	<i>C. tetanomorphum</i> 288	25		41		95
	<i>C. tetanomorphum</i> 500	30		31		88

* $\Delta T_{m(e)}$ is the difference between the temperatures at which there is 50% dissociation of homologous and heterologous DNA duplexes.

10% blood broth and incubated at 37 °C for 3 d. A loopful of 3 d-old culture was streaked on a PM plate and incubated anaerobically under hydrogen at 37 °C for 3 d. Small translucent colonies were successively subcultured on the same medium.

Test for toxigenicity. This was performed using PII medium according to the methods of Sanada & Nishida (1965) and Nishida *et al.* (1969).

RESULTS

DNA-DNA homology

On the basis of tests for DNA-DNA homology, *C. tetani* and its related strains were separated into three genetically related groups (Table 2). All of 10 *C. tetani* strains and three non-toxicogenic *C. tetani*-like strains were included in group I. Group II comprised *C. tetanomorphum* strains 1772 C, CIA, A and 2909, *C. cochlearium* strains montréal and 17787, and *C. lentoputrescens* type strain 17794. Group III comprised *C. tetanomorphum* strains 288, 500 and 543; these were distinct from the other two groups.

Further tests were performed to determine the thermostability of the duplexes formed between DNA from *C. tetani* HA-47 (group I) or *C. cochlearium* 17787 (group II) and DNA from some strains of groups I, II and III. The temperatures ($T_{m(e)}$) at which 50% of the DNA duplexes between the group II strains and *C. cochlearium* 17787 dissociated were similar to those of homologous DNA duplexes of *C. cochlearium* 17787 ($\Delta T_{m(e)}$ 0 to 0.5). The $T_{m(e)}$ value for the DNA duplexes between *C. tetanomorphum* 543 (group III) and *C. cochlearium* 17787 was lower, by 13.3 °C, than that of homologous DNA duplexes of *C. cochlearium* 17787. These data again confirmed that *C. cochlearium*, *C. lentoputrescens*

Table 3. *Biochemical properties for differentiation*

Biochemical property	Group based on DNA-DNA homology†		
	I	II	III
<i>Class A*</i>			
Fermentation of:			
Glucose	8/13‡	8/8	3/3
Inositol	12/13	0/8	3/3
Galactose	0/13	8/8	3/3
Glycerol	0/13	7/8	1/3
Ribose	0/13	7/8	3/3
Xylose	0/13	3/8	3/3
Maltose	0/13	0/8	3/3
Cellobiose, fructose, salicin, sorbitol	0/13	0/8	3/3
Mannose, sorbose	0/13	0/8	2/3
Liquefaction of gelatin:			
2% (w/v)	13/13	6/8	0/3
10% (w/v)	12/13	0/8	0/3
<i>Class B*</i>			
Digestion of milk	0/13	0/8	0/3
Digestion of coagulated albumen	0/13	0/8	0/3
Growth at 0 °C	0/13	0/8	0/3
Production of lecithinase	0/13	0/8	0/3
Reduction of nitrate	0/13	0/8	0/3
Digestion of casein	2/13	2/8	0/3
Production of indole	6/13	5/8	2/3
Production of H ₂ S	13/13	8/8	3/3
Haemolysis of human blood	13/13	2/8	0/3
Production of toxin	10/13	0/8	0/3

* Properties of class A are adopted for differentiation between groups I, II and III and those of class B for differentiation of the three groups from other associated clostridia.

† See Table 2.

‡ No. of strains positive/no. of strains tested.

and some strains of *C. tetanomorphum* were homologous and that two different kinds of strains were included in the species *C. tetanomorphum*.

Biochemical properties

Biochemical properties were re-investigated in a search for differential criteria corresponding to the genetic differences among the above-mentioned three groups and also for discriminating them from other associated species (Table 3). As the fermentation of most sugars by *C. tetani* and *C. tetani*-like strains was negative when read with a pH indicator, we measured the pH differences between cultures with and without sugar using a pH meter. Sugar fermentation tests were performed four times to confirm the validity of weak reactions.

Group I. Seven of these 13 strains fermented glucose and inositol. The Δ pH between 7 d cultures with and without glucose or inositol reached 1.95 or 1.6, respectively, as a maximum, but the final pH of these 7 d cultures with each sugar added only rarely fell below 6.0. These organisms fermented none of the remaining 28 sugars tested. Gas-chromatographic analysis of cultures in PY medium showed major peaks for butan-1-ol, acetic, propionic, butyric and lactic acids. The three non-toxigenic *C. tetani*-like strains were identified as *C. tetani* on a basis of DNA-DNA homology and biochemical properties.

Group II. No distinct differences in cultural and biochemical properties could be found among strains of this group, even though they were designated by different species names,

i.e. *C. cochlearium*, *C. tetanomorphum* and *C. lentoputrescens*. Spores could be detected microscopically in all strains except strain 2909. *Clostridium lentoputrescens* 17794 produced abundant spores. Other strains produced very few. Although spores were spherical to oval in all strains, spherical spores dominated in strains 17794, K-130 and Montréal.

All strains except 2909 and A attacked glucose, galactose, ribose and glycerol. The Δ pH in 7 d cultures with and without glucose or galactose ranged between 0.6 (strain 2909) and 2.1 (strain K-130) or between 0.5 (strain 1772 C) and 1.1 (strain A), respectively. The final pH of 7 d cultures with glucose fell below 6.0 in all strains except 17787 and 2909, but that of cultures with galactose did not. The Δ pH in 7 d cultures with and without glycerol reached 1.5 in strain CIA, but the final pH of these cultures rarely fell below 6.0. The Δ pH in 7 d cultures with and without ribose reached 1.32 (strain CIA) and the final pH, when Δ pH was over 0.5, fell below 6.0. The pH of uninoculated P medium with ribose, however, dropped to about 6.7, which was about 0.6 lower than that of uninoculated P medium with other sugars added.

Six strains of this group including the type strains of *C. cochlearium* and *C. lentoputrescens* liquefied gelatin.

Gas-chromatographic analysis of cultures in PY medium showed major peaks for acetic, propionic, butyric and lactic acids.

Group III. All of these three *C. tetanomorphum* strains fermented cellobiose, fructose, galactose, inositol, ribose, salicin, sorbitol and xylose, as well as glucose and maltose. None gave rise to pH values below 6.0 in the presence of sugars other than glucose, maltose, inositol and ribose. Mannose, sorbose and glycerol were fermented by some but not all strains. None of the remaining 17 sugars tested was fermented by the group III strains.

Gas-chromatographic analysis of cultures in PY medium showed major peaks for butan-1-ol, acetic, propionic and butyric acids.

Isolation of group II strains

Thirteen isolates from horse faeces, when tested for cultural and biochemical properties, were similar to the type strains of *C. lentoputrescens* or of *C. cochlearium*. Differential biochemical properties of these isolates are shown in Table 4. All of the isolates consistently and abundantly produced spherical spores, and seven of the 13 isolates exhibited weak but definite ability to liquefy gelatin in the test performed after 7 d incubation. It seems, therefore, justified to identify these isolates as *C. lentoputrescens*, but DNA-DNA homology studies on four of the isolates showed them to be genetically homologous with *C. cochlearium* type strain ATCC 17787 (Table 5).

DISCUSSION

Takahashi *et al.* (1967) demonstrated that two distinct groups of strains were included under the species name of *C. tetanomorphum*; one group comprised strains 1772 C, CIA, EC'1, A and NCTC 2909 and the other, strains NCTC 288, 500 and 543 and ATCC 15920 and 3606. The cultural and biochemical properties of the former group agree with those of *C. tetanomorphum* originally described as *Pseudotetanus bacillus* No IX by McIntosh *et al.* (1917). In the present tests for DNA-DNA reassociation, thermostability of DNA-DNA hybrids and biochemical properties, we demonstrated that *C. tetanomorphum* strains 1772 C, CIA, A and NCTC 2909 were genetically homologous with the *C. cochlearium* type strain ATCC 17787 as well as with *C. cochlearium* strains from the Pasteur Institute. *Clostridium tetanomorphum* strains NCTC 288, 500 and 543 were distinctly different from *C. cochlearium* and *C. tetani* strains in DNA-DNA homology as well as in cultural and biochemical properties. These strains were isolated from wounds in 1921 by M. Robertson (private communication from S. Lapage, NCTC) and have been used since then as the representative strains of this species. In the present study they attacked numerous sugars but the pH of the

Table 4. *Differential biochemical properties of isolates from horse faeces*

Biochemical property	Isolates
Fermentation of:	
Glucose, glycerol	13/13*
Ribose	12/13
Galactose	9/13
Inositol, xylose, maltose, cellobiose, fructose, salicin, sorbitol, mannose, sorbose	0/13
Liquefaction of gelatin:	
2% (w/v)	7/13
10% (w/v)	0/13

* No. of strains positive/no. of strains tested.

Table 5. *Similarities between DNA from isolates from horse faeces and DNA from reference strains C. cochlearium ATCC 17787 and C. tetani HA-47*

Organism	% Homology with DNA from:	
	ATCC 17787	HA-47
<i>C. tetani</i> HA-47	64	100
<i>C. cochlearium</i> ATCC 17787	100	56
Isolate H-4	92	59
Isolate H-8	92	59
Isolate H-7	92	59
Isolate no. 20	91	60

7 d cultures did not fall below 6.0 in the presence of sugars other than glucose, maltose, inositol or ribose.

In the carbohydrate fermentation, we used pH measurement whereas most diagnostic and identification studies involve pH indicators. Thus, many of the reactions reported here as positive would be negative using pH indicators as the pH frequently did not fall below 6.0. Fermentation results of this group, therefore, agree with the original description of *C. tetanomorphum* (Bulloch *et al.*, 1919). Although Fildes, together with McIntosh *et al.* (1917), isolated different kinds of non-toxicogenic *C. tetani*-like strains which were later established as *C. tetanomorphum* and *C. cochlearium* by their colleagues (Bulloch *et al.*, 1919), Fildes (1927) stated that the two species as well as *C. lentoputrescens* were not worthy of separation, being at best merely representatives of the saccharolytic extreme on the one hand or proteolytic on the other. Our present study has verified the validity of Fildes' viewpoint on the relationship between *C. cochlearium* and some strains of *C. tetanomorphum* and has also revealed that the type strain and our isolates of *C. lentoputrescens* are genetically homologous with a type strain of *C. cochlearium*. As the two species' names *C. tetanomorphum* and *C. cochlearium* were simultaneously established by the same authors (Bulloch *et al.*, 1919), neither has precedence. *Clostridium tetanomorphum* strains in group II that are genetically homologous with the type strain of *C. cochlearium* should be renamed as *C. cochlearium* and the species name *C. tetanomorphum* kept for strains NCTC 288, 500 and 543 as well as for strains ATCC 3606 and 15920.

One of the main criteria differentiating *C. lentoputrescens* from *C. cochlearium* is the ability to liquefy gelatin. Using 2% (w/v) gelatin or the method of Holdeman & Moore (1975), we found that most *C. cochlearium* strains attacked gelatin. Hartsell & Rettger (1934) suggested the identity of *C. lentoputrescens* with *C. cochlearium* from various authors, when they proposed the name *C. lentoputrescens*. The species name *C. lentoputrescens* should be abandoned as a later synonym of *C. cochlearium*. All of our isolates previously designated as *C. lentoputrescens* were obtained from horse faeces samples. Although Jawetz *et al.* (1976)

stated that *C. tetani* could frequently be isolated from horse faeces and Smith (1975) referred to *C. tetani* in horse manure, we have seldom isolated *C. tetani* strains from horse faeces in spite of the frequent presence of *C. tetani*-like spores in the samples. Fildes' method (Fildes, 1927) was ineffective for isolating anaerobes with *C. tetani*-like spores, since they did not exhibit swarming.

The name *C. pseudotetanicum* (Prévot) comb. nov. Smith & Hobbs 1974, has been listed in the eighth edition of *Bergey's Manual*. Nakamura *et al.* (1973), however, demonstrated that a *Plectridium pseudotetanicum* strain tested was in agreement with *C. tertium* in its cultural and biochemical properties.

Another *C. tetani*-like species fermenting glucose and maltose, *C. tetanoides* (Adamson) Hauduroy 1937, is described in the seventh edition of *Bergey's Manual*. No reliable strains of *C. tetanoides* or of *C. pseudotetanicum* are available, however, and we feel justified in not yet recognizing these species as definite entities.

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