JOURNAL OF BACTERIOLOGY, Mar., 1965
Vol. 89, No. 3

Isolation of *Clostridium tetani* from Soil

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Received for publication 6 October 1964

ABSTRACT

SANADA, ICHIRO (Kanazawa University, Kanazawa, Japan), AND SHOKI NISHIDA. Isolation of *Clostridium tetani* from soil. J. Bacteriol. 89:626–629. 1965.—The higher the temperatures applied to soil specimens, the weaker the toxigenicity of *Clostridium tetani* strains isolated from them. The glucose- and maltose-fermenting ability of these isolates was inversely proportional to their toxigenicity. The biological properties of atoxic strains were indistinguishable from those of *C. tetanomorphum*. Since a considerable number of toxic strains fermented glucose and maltose, these criteria are of doubtful value for differentiating *C. tetani* from *C. tetanomorphum*.

It has been demonstrated in the isolation of clostridia from soil that strains of a given species vary in toxigenicity (Yamagishi, Ishida, and Nishida, 1964; Nishida, Tamai, and Yamagishi, 1964). Existence of nontoxigenic strains, as well as strains with different degrees of toxigenicity, seems to be plausible, but nontoxigenic strains are likely to be left unidentified or may be classified into nontoxigenic species of similar biological nature. Since the presence of toxin is regarded as the most important criterion for identifying toxigenic species of clostridia, and since conversion of toxigenic to nontoxigenic strains or vice versa has not been demonstrated, the classification based on specificity of toxin is considered to be preferable to other taxonomic characters because of its practical importance (Smith, 1955; Oakley, 1962). However, in the light of recent findings in our laboratory that the loss of toxigenicity in clostridia is in general closely related to increased sporulating ability, and the finding of Zeissler (1930) that *Clostridium tetani* is weaker in sporulating ability than *C. tetanomorphum*, an investigation was undertaken to elucidate the relationships among toxigenic and nontoxigenic strains of *C. tetani* and *C. tetanomorphum*.

To obtain strains of *C. tetani* possessing a variety of sporulating abilities, we attempted isolation from soil specimens heated at various temperatures, according to the method of Yamagishi et al. (1964). The toxigenicity and the biological nature of 87 isolates were analyzed.

MATERIALS AND METHODS

Isolation. Cultures were isolated by the method of Fildes (1925) from soil samples collected in Kanazawa. A soil specimen weighing approximately 2 g was added to 10% blood broth, and was then heated at various temperatures. The sample was cooled immediately after heating, and was then incubated for 4 days; 0.5-ml portions of the 4-day-old cultures were carefully transferred into the bottom of agar slants without touching the surface of the medium. Since *C. tetani* swarms rapidly, typically swarming colonies developing on the top of the slant in 3 to 5 days were smeared onto Zeissler's agar plates and were incubated anaerobically. Colonies agreeing in colonial morphology with the description of Zeissler (1930) were transferred to cooked meat broth, and the cultures were examined for biological activity and sporulating ability.

Medium for toxin production. Taylor's (1945) digest medium was used at the beginning of this experiment. Later, papain digest peptone (Daigo Co., Osaka, Japan) broth was used; 10 ml of a broth containing peptone (40 g/liter) and NaCl (5 g/liter) were adjusted to *p*H 7.2 and distributed into test tube (15 by 150 mm). Sodium thioglycolate and glucose were added to a concentration of 0.1 and 0.5%, respectively, before inoculation. The cultures were incubated for 4 days in an anaerobic jar.

Estimation of toxigenicity. The 4-day-old culture was centrifuged at 2,000 × *g* for 30 min. Toluene was added to about one-fifth volume of the supernatant fluid, and the mixture was frequently shaken. Toxigenicity was examined the following day after removal of the toluene by filtration. Tenfold dilutions of the supernatant fluid in 1% peptone-water were prepared, and 0.5 ml of each dilution was injected into the hind leg of each of two mice. The reciprocal of the maximal dilution which killed both mice in 3 days is expressed as the MLD/0.5 ml.

Biological criteria for identification. Other criteria were examined by the routine procedure adopted in our laboratory (Nishida et al., 1964). Considering the importance of peptone as an ingredient in the basal medium, we examined

ten brands of peptone: Poli-, Papain digest-, and Kongo-peptone (Daigo Co., Osaka, Japan); Proteose- and Neo-peptone (Difco); Arei peptone (Kyoei Co., Tokyo, Japan); Tryptone and Shokubutsu peptone (Nichiei Co., Tokyo, Japan); Mikuni peptone (Mikuni Co., Tokyo, Japan); and Kyokuto peptone (Kyokuto Co., Tokyo, Japan). All cultures were negative in fermentation of maltose, lactose, and sucrose and in digestion of coagulated egg albumen. However, discrepancy was observed in glucose fermentation and, to a lesser extent, in gelatinolysis. We identified tetanus toxin-producing strains as *C. tetani* regardless of these discrepancies. The tetanus toxin was identified by its production of typical symptoms in mice and its successful neutralization by tetanus antitoxin. The nontoxigenic strains which were closely similar to *C. tetani* in their biological properties, as well as in their colonial and microscopical morphology, were also identified as such in spite of their discrepancies in glucose fermentation and gelatinolysis. There may be, of course, some controversy about the relation of nontoxigenic strains of *C. tetani* to *C. tetanomorphum*, and this will be discussed more completely below.

RESULTS

Effects of temperatures on toxigenicity. Isolation was attempted several times from 304 soil samples heated at various temperatures: 60, 80, and 90 C for 10 min, and 100 C for 30 min (Table 1).

From 125 soil samples heated at 60 C for 10 min, 43 cultures of *C. tetani* were isolated. The toxigenicity of these cultures ranged between 0 and 10^5 MLD/ml of culture filtrate, and most cultures displayed a toxigenicity of 10^2 to 10^5 MLD/ml. Of 30 soil samples heated at 80 C for 10 min, 12 (40%) produced cultures. Their toxigenicity ranged between 0 and 10^3 MLD/ml. The average level of toxigenicity was far lower than that of the former group. From 48 samples heated at 90 C for 10 min, 11 isolates (22.9%) were obtained. Their toxigenicity was approximately the same as that of the first (60 C) group. From 66 samples heated at 100 C for 10 min, 18 (27.3%) produced cultures; from 35 samples heated at 100 C for 30 min, only 3 cultures (8.5%) were isolated. Only 2 of these 21 isolates were toxigenic (10 MLD/ml).

TABLE 1. *Toxigenicity of Clostridium tetani isolated from soil specimens heated at various temperatures*

No. of specimens	Temp	No. of strains isolated	Toxigenicity (MLD/ml)						
			10^5	10^4	10^3	10^2	10^1	10^0	0
	C								
125	60	43 (34.4%)	4	3	12	19	2	0	3
30	80	12 (40.0%)	0	0	1	2	2	1	6
48	90	11 (22.9%)	0	0	3	0	3	1	4
66	100	18 (27.3%)	0	0	0	0	1	0	17
35	100*	3 (8.6%)	0	0	0	0	1	0	2

* Heat applied for 30 min, rather than the usual 10 min.

These findings suggest that the higher the temperature for preheating samples, the lower the toxigenicity (and the fewer toxigenic strains isolated). It would appear that toxigenic strains are more susceptible to heat than are nontoxigenic strains of *C. tetani*.

TABLE 2. *Relation of biological type to toxigenicity in 87 strains*

Toxigenicity (MLD/ml)	Biological type*				Ratio of glucose-fermenting strains
	I	II	III	IV	
10^5	0†	4	0	0	0/4‡
10^4	0	2	1	0	1/3
10^3	3	11	2	0	2/16
10^2	1	14	3	3	6/21
10^1, 10^0	0	6	4	1	5/11
0	2	9	15	6	21/32

* See text for definition of types.

† Figures indicate the number of strains showing the corresponding biological type at each toxic level.

‡ Denominator indicates the total number of strains at each toxigenicity level; numerator indicates glucose-fermenting strains.

Biological criteria for identifying C. tetani. Eight biological criteria were used to test the 87 isolates. Variation was found only in glucose fermentation and gelatinolysis (Table 2). The cultures were grouped into four biological types: type I (glucose fermentation, −; gelatinolysis, −); type II (−, +); type III (+, +); and type IV (+, −). Type I is characteristic of *C. cochlearium*, type II of *C. tetani*, and type IV of *C. tetanomorphum*. There have been no previous descriptions of type III; we assume this type to be a form intermediate between type I and type II, for the reasons described below.

Toxigenicity was strong in most type II strains, and weak or negative in strains of type IV; type III strains showed an intermediate behavior in their toxigenicity. The strains of type I also displayed weaker toxigenicity than those of type II. Strains of type I and type IV were infrequently isolated. This also indicates that the weaker the toxigenicity, the greater the likelihood of obtaining glucose-fermenting strains. Glucose fermentation was positive in 24.4% of 55 toxigenic strains, and gelatinolysis was negative in 14.5%.

All of nontoxigenic glucose-fermenting strains, although they were extremely similar to *C. tetanomorphum*, were negative in maltose fermentation. We suspected, however, that the negative result might be due to some cultural condition employed in our laboratory. This was investigated from two aspects: kinds of peptone used, and the dates of reading results. Ten brands of peptone were used for preparing the basal medium for fermentation. Instead of reading the results at the end of the 7th day of cultivation, we read the results on the 3rd, 5th, and 7th days; triplicate tube cultures were examined one by one on these days. Two strains each of the toxigenic and nontoxigenic groups were used. Glucose fermentation was also examined under the same conditions.

Maltose fermentation. Two toxigenic strains did not show acid production at any time (Table 3). One nontoxigenic strain was positive in 6 of 10 peptone media when read on the 7th day. The other strain was negative in all kinds of peptone.

Glucose fermentation. Both strains of the toxigenic group produced acid in almost all peptone media when examined on the 3rd and 5th days, but the reaction was negative in some peptone media when examined on the 7th day. Nontoxigenic strains differed in their behavior. One strain displayed acid production in almost all media throughout the period of examination, but another strain showed negative results in some of the peptone media when examined on the 7th day of incubation. These findings again demonstrate that glucose-fermenting activity can not be used as a criterion for differentiating *C. tetani* from *C. tetanomorphum*.

Biological variation in 57 strains. Further experiments were carried out by use of two peptone media (Proteose and Arei) and 57 strains possessing various degrees of toxigenicity. Acid production was tested every day for 7 days by estimating transitional changes of *p*H in each tube culture with *p*H indicator paper (Toyo Filter Paper Co., Tokyo, Japan). The reaction was regarded as positive whenever the initial *p*H (7.2) fell to 6.6 or lower. The 57 strains were arranged into three biological types: type I (glucose fermentation, −; maltose fermentation, −); type II (+, −); and type III (+, +). Type I is characteristic of *C. tetani* and type II of *C. tetanomorphum*. There have been no previous descriptions of type III. The results (Table 4) again verify that the number of glucose- and maltose-fermenting strains increased as the toxigenicity of the strains decreased. When Arei peptone was used, 82.3% of the nontoxigenic strains were maltose-positive, thus revealing that these strains are similar to *C. tetanomorphum*.

Gelatinolysis activity was examined in the same two peptone media. A great difference was noted in the results (Table 5) obtained. These findings were also markedly different from those obtained in Poli peptone medium (Table 2). In Arei peptone medium, most strains were gelatinolysis-negative.

TABLE 3. *Biological test for fermentation in various peptone media*

Fermentation	Strain*	Incubation period (days)		
		3	5	7
Glucose	HA-47	10/10†	10/10	5/10
	H-1	9/10	10/10	3/10
	T-6139	10/10	10/10	10/10
	T-1101	9/10	10/10	5/10
Maltose	HA-47	0/10	0/10	0/10
	H-1	0/10	0/10	0/10
	T-6139	10/10	9/10	6/10
	T-1101	1/10	0/10	0/10

* Strains HA-47 and H-1 are toxigenic strains; strains T-6139 and T-1101 are nontoxigenic.

† Number of positive reactions per number of peptone media employed.

TABLE 4. *Relation of biological type to toxigenicity in 57 strains*

Toxigenicity (MLD/ml)	Biological type* (Proteose peptone)			Biological type (Arei peptone)		
	I	II	III	I	II	III
10^4	1†	1	0	1	1	0
10^3	11	3	2	8	6	2
10^2	3	2	1	2	2	2
10^1	4	0	1	4	0	1
10^0	1	3	1	0	0	5
0	4	16	3	2	2	19

* Types defined in text.

† Figures indicate the number of strains showing corresponding biological types at each toxigenic level.

TABLE 5. *Gelatinolysis-negative strains in different peptone media*

Toxigenic level of strains (MLD/ml)	Peptone used	
	Proteose	Arei
10^4 to 10^3	12/18* (55.6%)	17/18 (84.4%)
10^2 to 10^0	6/16 (37.5%)	13/16 (81.2%)
0	16/23 (69.6%)	21/23 (81.3%)

* Number of gelatinolysis-negative strains per total strains at each toxigenic level.

DISCUSSION

Fildes (1925) stated that gelatinolysis is inadequate for identifying *C. tetani* because of its variability. He also showed that many strains of *C. tetani* are negative in gelatinolysis. The variability in gelatinolysis seems to be also true of *C. tetanomorphum*. Hall (1922), however, claimed that all strains of *C. tetanomorphum* he examined were positive in this reaction.

Nontoxigenic strains were usually isolated from samples heated at higher temperatures. Since Zeissler (1930) and Sasaki (1933) have shown that *C. tetanomorphum* has greater sporulating ability than *C. tetani*, the present results suggest that the nontoxigenic strains should be identified as *C. tetanomorphum*. *C. cochlearium*-like strains also seem to be worthy of reinvestigation, because some of these strains can produce tetanus toxin. Gelatinolysis also seems to be of doubtful value as a criterion for distinction between *C. tetani* and *C. cochlearium*, although this is the only differential taxonomic criterion, other than toxigenicity, now used. We leave this taxonomic problem for future investigation.

The postulation that *C. tetanomorphum* might be highly sporulating strains of *C. tetani* recalls the claim of Nishida et al. (1964) and Tamai and Nishida (1964) that *C. bifermentans* is a highly sporulating strain of *C. sordellii*. The relationship of sporulating potency to toxigenicity and other taxonomic characters needs further investigation.

Though cultures of *C. tetani* do not show acidity in the presence of any carbohydrate, many investigators have reported that this organism can utilize glucose (Reddish and Rettger, 1924; Lerner and Pickett, 1945; Martinez and Rittenberg, 1959). We, however, demonstrated that many strains of *C. tetani* can produce acid from glucose. This finding does not agree with present taxonomic opinion. We believe that the discrepancy is due mainly to the difference between our cultural conditions and those employed by others. We used media adequately prepared for potent toxin production, so that we could easily isolate weakly toxigenic and glucose-fermenting strains of *C. tetani;* these cultures could then be identified as nontoxigenic species when examined in the usual media.

ACKNOWLEDGMENT

We express our appreciation to C. T. Huang of Hong Kong University for his helpful advice, and to L. DS. Smith of Montana State College for his instructive criticism on this manuscript.

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