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TAXONOMY OF *CLOSTRIDIUM BIFERMENTANS* AND *CLOSTRIDIUM SORDELLII*

II. Toxigenic and Sporulating Potencies in Substrains of a *Clostridium sordellii* Strain

KENZO TAMAI and SHOKI NISHIDA

Department of Bacteriology, School of Medicine, Kanazawa University, Kanazawa, Japan

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Abstract

Tamai, Kenzo (Kanazawa University, Kanazawa, Japan), and Shoki Nishida. Taxonomy of *Clostridium bifermentans* and *Clostridium sordellii*. II. Toxigenic and sporulating potencies in substrains of a *Clostridium sordellii* strain. J. Bacteriol. **88**:1647–1651. 1964.—The existence of six biological criteria for distinction of *Clostridium bifermentans* and *C. sordellii* was confirmed. The difference in the six criteria gradually disappeared as the sporulating potency of the substrains of *C. sordellii* 4708 was strengthened. The substrains which could resist heating at 90 C for 10, 20, or 30 min were found to have lost all six criteria for distinction and were biologically in agreement with *C. bifermentans*. We further demonstrated that all newly isolated strains of *C. bifermentans* examined possessed extremely strong sporulating potency.

In the preceding report (Nishida, Tamai, and Yamagishi, 1964), we demonstrated that the sporulating potency of *Clostridium bifermentans* was distinctly stronger than that of *C. sordellii* and that *C. bifermentans*-like substrains could be obtained by selecting heat-resistant substrains from parent strains of *C. sordellii*. Recently, Brooks and Epps (1959) disclosed that these species could be distinguished from each other by many biological activities in addition to the two main distinctions, namely, urease activity and toxigenicity. The existence of more biological distinctions makes possible more exact identification of the species; therefore, a study was undertaken to determine whether the above-mentioned *C. bifermentans*-like substrains of *C. sordellii* could be identified as *C. bifermentans* by these additional criteria. In other words, we attempted in this study to determine whether or not *C. bifermentans* can be obtained from *C. sordellii*.

Materials and Methods

Strains used. The nature of the 13 strains of *C. sordellii* employed was described in the preceding paper; 13 newly isolated strains of *C. bifermentans* were used in conjunction with the 12 strains of this species previously described.

Biological test. The test was carried out according to the description of Sterne and van Heyningen (1958). Sugar-fermenting activity was determined by adding 0.2 ml of a 1% solution of methyl red-bromothymol blue reagent (Nishida et al., 1964) to each of the broth cultures which had been incubated for 7 days. Indole formation was examined by a routine method. A 0.5-ml amount of a 50% (v/v) solution of H_2SO_4 and 0.5 ml of amyl alcohol solution saturated with KNO_2 were added to 2.5 ml of the cultural fluid. The mixture, after sufficient shaking, was left standing for a short while. Positive reaction corresponding to the strength of indole produced was indicated by variation from pinkish to deep-red color.

Proteolytic activity. Proteolytic activity was measured by Brooks and Epps' (1959) method. To eliminate the sterile procedure employed in this method, we modified it as follows. A 24-hr chopped-meat broth culture was centrifuged, and the supernatant fluid was diluted twofold. A 1-ml amount of each dilution was mixed with an equal volume of a 10% solution of gelatin in a small test tube (13 by 100 mm). After the mixture was incubated at 37 C for 4 hr, it was chilled to observe the presence or absence of gelatinolysis. The maximal dilution causing gelatinolysis was determined after a few hours.

Heat-resistance test or estimation of sporulating potency was described in the previous paper.

TABLE 1. *Sugar-fermenting activity of Clostridium sordellii and C. bifermentans in various peptone broths*

Sugar	Strain used	Peptone employed					
		Proteose (Daigo)	Poli (Daigo)	Arei (Kyoei)	Proteose (Difco)	Daizu (Daigo)	Soitone (Difco)
Sorbitol*	*C. sordellii* (pathogenic)	1/8†	7/8	7/8	7/8	5/8	5/8
	C. sordellii (nonpathogenic)	0/5	3/5	3/5	2/5	1/5	1/5
	C. bifermentans	7/12	8/12	8/12	12/12	8/12	6/12
Mannose‡	*C. sordellii* (pathogenic)	1/8	8/8	8/8	2/8	1/8	5/8
	C. sordellii (nonpathogenic)	1/5	5/5	5/5	1/5	2/5	2/5
	C. bifermentans	11/12	12/12	12/12	12/12	11/12	12/12

* In most positive cases, acid production by strains of both species was extremely weak.

† Number of fermentation-positive strains per number of strains employed.

‡ The color reaction due to acid production was distinctively different between *C. sordellii* and *C. bifermentans*.

RESULTS

Since the biological nature of clostridia varied a great deal according to the cultural conditions employed, and consequently results obtained by different investigators were not always the same, we attempted to examine the findings of Brooks and Epps (1959) on the biological nature of *C. bifermentans* and *C. sordellii* under the conditions employed in our laboratory.

As previously described, the results in toxigenicity and urease activity of *C. sordellii* strains we had employed were in complete agreement with those shown by Brooks and Epps (1959). In this paper, therefore, only the other biological distinctions were investigated.

Colonial morphology. Colonial morphologies of *C. sordellii* and *C. bifermentans* on Zeissler's agar were in agreement with those described by Brooks and Epps (1959). In this study, the typical colony of *C. bifermentans* was designated as an S-type colony, and the typical colony of *C. sordellii* was designated as an R-type colony. Furthermore, the intermediate type of colonies, which were not identical to either of them but possessed some resemblance to each species, was designated as S-R type.

Sorbitol fermentation. We could not find a distinction in sorbitol-fermenting activity between the two species. Since the pH of the fermentation medium on the seventh day of incubation is significantly influenced by the nature of peptone employed, eight brands of peptone were examined. Our findings seemed to approximate those of Brooks and Epps (1959), providing proteose peptone was used. However, the positive reaction due to acid production was extremely weak and could not be used as a definite criterion for differentiation (Table 1).

Mannose fermentation. When a proper peptone, such as proteose peptone (Daigo Co., Osaka, Japan, or Difco), was used, mannose fermentation was useful in differentiation of *C. sordellii* from *C. bifermentans* (Table 1).

Esculin and salicin fermentation. Preliminary tests for fermentation of esculin and salicin were performed. Judging from the unsuccessful findings obtained, we omitted these sugars from the criteria for differentiation.

Proteolytic activity. The proteolytic activity of both species was estimated by Brooks and Epps' (1959) method as well as by ours. The proteolytic activity of *C. bifermentans* was distinctively stronger than that of *C. sordellii* (Table 2). The activity of the *C. sordellii* N-P group was more like that of *C. sordellii* than like that of *C. bifermentans.*

Indole formation. Although Brooks and Epps (1959) did not employ indole-forming activity as a differential between these two species, we could observe a distinctive feature in this activity

(Table 3). Further confirmation of indole-forming activity of five strains each from the two species was carried out for 3-, 5-, and 8-day-old cultures. During the course of the above experiment, two brands of peptone were used. When examined at any time in either peptone employed, color reaction due to indole production was distinctive between cultures of the two species. Thus, we found that in addition to the two principal criteria for differentiation, urease activity and toxigenicity, other criteria such as colonial morphology, mannose fermentation, proteolytic activity on gelatin, and indole production can be used to differentiate *C. bifermentans* from *C. sordellii*.

Differences in sporulating potency and biological activity of substrains of a strain of C. sordellii. The relationship between the heat-resistant nature and the biological nature mentioned above was analyzed with substrains of *C. sordellii* 4708. A 1-ml amount of a 24-hr chopped-meat broth culture of each organism was transferred into six small test tubes. Careful attention was given to avoid concomitant transferral of chopped-meat particles. The six cultural suspensions in the sterile test tubes were heated at 80 C for 10, 20, and 30 min, and at 90 C for 10, 20, and 30 min. Immediately after heating, 0.5 ml from each of the heated suspensions was transferred into fresh chopped-meat broth, and the cultures were incubated for 48 hr. The heat-resistant substrains from each culture were examined for the above criteria. Their sporulating potency was also examined. Two strains of *C. sordellii* (1694 and 3703) and two strains of *C. bifermentans* (317 and 4701) were used as the standard controls. On the plate culture of a substrain of *C. sordellii* 4708, which could resist 80 C for 10 or 20 min, S-type colonies could be found among the typical R-type colonies, but this strain did not display any variation in biological activities from the parent strain. The substrain resistant to 80 C for 30 min turned out to be urease-negative and atoxic, but indole production and mannose fermentation were still those of *C. sordellii*. The substrain resistant to 90 C for 10 min presented the indole-forming activity of *C. bifermentans* and fermented mannose, although the fermentative activity was far weaker than that of *C. bifermentans* (Table 4).

TABLE 2. *Proteolytic activities of Clostridium sordellii and C. bifermentans*

Strain used	Proteolytic activity	
	Brooks and Epps' method	Modified method
C. sordellii (pathogenic)		
4709	8*	1†
4707	4	1
1732	4	0
1734	32	8
1733	8	0
C. sordellii (nonpathogenic)		
6929	8	0
1619	4	0
1620	8	0
1621	2	0
1623	2	0
C. bifermentans		
7103	128	8
317	64	8
315	128	8
7033	128	8
7038	64	8

* The figures indicate maximal dilutions of cultures to cause gelatinolysis.

† The figures show the maximal dilutions of culture filtrates to cause gelatinolysis.

TABLE 3. *Indole-producing activities of Clostridium sordellii and C. bifermentans**

Organism	No. of strains used	Indole production†			
		−	±	+	++
C. bifermentans........	25	0	12	12	1
C. sordellii............	13	0	0	2	11

* Peptone used: proteose peptone (Daigo).

† Symbols: ++ = red color reaction; + = pink color reaction; ± = slightly pink color reaction; − = no color reaction.

The proteolytic activity of the culture filtrate of a substrain which could resist 90 C was estimated in conjunction with the parent strain and a strain of *C. bifermentans* (317) by our method. The parent strain displayed no gelatinolysis, but its heat-resistant substrain turned out to be positive in this reaction, although the activity was far weaker than that of *C. bifermentans* 317 (Table 4).

Another heat-selection test was performed for *C. sordellii* 4708 in a slightly different manner. A 24-hr chopped-meat broth culture of parent strain was distributed into six tubes (13 by 100 mm), and each tube was heated to various tem-

TABLE 4. *Biological nature and proteolytic activity of heat-resistant substrains of Clostridium sordellii 4708*

Strain used	Urease reaction	Toxigenicity	Indole reaction	Mannose fermentation	Colonial type	Proteolytic activity*
Prototrophic strain	+	+	++	−	R	0
Substrains resistant to 80 C for 10 min	+	+	++	−	R, S	
80 C for 20 min	+	+	++	−	R, S	
80 C for 30 min	−	−	++	−	R, S, R-S	
90 C for 10 min	−	−	+	+†	R, S, R-S	
90 C for 20 min	−	−	+	+†	R, S, R-S	
90 C for 30 min	−	−	+	+†	R, S, R-S	2
C. bifermentans 317						8

* The figures show the maximal dilution of culture filtrates to cause gelatinolysis by our method.
† Acid production was slightly positive.

TABLE 5. *Sporulating potency of substrains of Clostridium sordellii 4708 of different biological natures*

Substrains used	Biological criteria*					Sporulating potency†	
	Ur	Tox	Ind	Man	Col	No. of total grown cells per ml	No. of heat-resistant cells per ml
Prototrophic strain	+	+	++	−	R	2.4×10^8	7.8×10^1
Substrain resistant to							
80 C for 20 min	+	+	+	−	S	3.3×10^8	4.5×10^3
80 C for 30 min	+	−	+	−	S	3.3×10^8	6.8×10^4
90 C for 30 min	−	−	+	+‡	S	6.8×10^8	3.3×10^7

* Ur, urease activity; Tox, toxigenicity; Ind, indole production; Man, mannose fermentation; Col, colonial morphology.
† The sporulating potency of a standard strain of *C. bifermentans* 317 was proven to contain 9.3×10^7 heat-resistant cells per 2.7×10^8 total grown cells per ml.
‡ Slightly positive.

TABLE 6. *Sporulating potencies of newly isolated Clostridium bifermentans*

Strain no.	Sporulating potency	
	No. of total grown cells per ml	No. of heat-resistant cells per ml
2	3.5×10^8	1.7×10^8
3	7.9×10^8	1.7×10^8
4	2.8×10^8	2.4×10^8
5	4.9×10^8	3.3×10^8
6	1.3×10^8	1.1×10^8
7	2.4×10^8	1.3×10^8
8	7.9×10^7	1.3×10^7
10	1.7×10^8	1.1×10^8
Standard strain *C. bifermentans* 317	4.9×10^8	1.1×10^8

peratures as prescribed. After heating, 0.4 ml of each of the heated suspensions was directly plated onto a Zeissler's blood-agar plate. When the culture fluid was absorbed by the blood-agar, the plate was incubated at 37 C for 48 hr. From the plate subcultures, S and S-R types of colonies were removed, and their biological activities were examined. The results obtained were more or less similar to those found in the preceding experiment except that, when the substrains were plated again onto Zeissler's agar medium, colonies of the same type of colonial morphology were obtained.

Since the above procedure suggests that the higher the temperature is, the stronger will be the sporulating potency of the substrains, quantitative estimation to correlate sporulating potency

of the substrains and their biological activities was carried out. Substrains possessing a biological nature distinct from those of the parent strain and other substrains possessing distinctive sporulating potency were found (Table 5). Substrains which acquired a sporulating potency equal to *C. bifermentans* were proven to be completely in agreement with *C. bifermentans* in biological characters too.

Sporulating potency of newly isolated C. bifermentans. An additional experiment to confirm our finding was conducted on the sporulating potency of newly isolated strains of *C. bifermentans*. Eight strains were tested and were again found to possess distinctively high ratios of sporulating potency (Table 6).

DISCUSSION

Yamagishi et al. (1964), Nishida et al. (1964), and Sanada (1962) disclosed with other species of clostridia that most strains isolated from unheated specimens of soil possess extremely weak heat resistance. Considering our results in the preceding and the present papers, we assumed that *C. sordellii* might be isolated from unheated specimens, although the isolation of this organism from soil has rarely been reported to date. We, therefore, attempted to isolate heat-nonresistant strains of *C. bifermentans* from unheated soil specimens or animal feces. From these samples, unheated or sometimes heated at a low temperature, we could easily isolate *C. bifermentans* to an extent of 260 positive cultures in 300 samples. Eight strains arbitrarily selected were examined for their sporulating and toxigenic potencies. Regardless of the unheated conditions, the eight strains were found to possess high sporulating potency and to be atoxic. In other words, the colonial characteristics of *C. bifermentans* seem to be descriptive for colonies comprised of cells possessing strong sporulating potencies, although the colonies on Zeissler's agar did not always contain sporulating cells when microscopically examined.

In this study, we succeeded in obtaining *C. bifermentans* from *C. sordellii* 4708. In another study, however, we could not obtain *C. bifermentans* or even a *C. bifermentans*-like substrain from another strain of *C. sordellii* by heat selection (*unpublished data*). Further investigation will be carried out to determine the factors and conditions which would enable easy selection of *C. bifermentans* from *C. sordellii*.

It must be noted that, in the determination of fermentation results of anaerobes, the biological process taking place during the incubation period is often neglected, because the pH indicator to detect change of reaction is usually added to the broth culture on the day of final reading (Sterne and van Heyningen, 1958; Willis, 1960), and the incubation period is not clearly shown in the existing textbooks. The diversity in biological activities of *C. sordellii*, as observed by us and by Brooks and Epps (1959), may also be due to differences in the cultural conditions, particularly in the type of peptone employed. It is desirable to standarize those conditions in the future.

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