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# TAXONOMY OF *CLOSTRIDIUM BIFERMENTANS* AND *CLOSTRIDIUM SORDELLII*

## I. Their Toxigenicity, Urease Activity, and Sporulating Potency

SHOKI NISHIDA, KENZO TAMAI, AND TAKAYOSHI YAMAGISHI

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

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### Abstract

Nishida, S. (Kanazawa University, Kanazawa, Japan), K. Tamai, and T. Yamagishi. Taxonomy of *Clostridium bifermentans* and *Clostridium sordellii*. I. Their toxigenicity, urease activity, and sporulating potency. J. Bacteriol. **88**:1641–1646. 1964.—Strains with properties similar to those of *Clostridium bifermentans* were usually obtained by selecting heat-resistant substrains of *C. sordellii* 1734. Heat-resistant substrains obtained from seven other strains were also found to be nontoxic. Some of these heat-resistant substrains produced urease, but others did not. Substrains of typical cultures of *C. sordellii* thus can yield either substrains resembling nonpathogenic strains of *C. sordellii* culturally, or substrains resembling *C. bifermentans*. The sporulating potency of pathogenic and nonpathogenic strains of *C. sordellii* and strains of *C. bifermentans* proved to be significantly distinct. The sporulating potency of *C. bifermentans* was high, that of pathogenic strains of *C. sordellii* was low, and that of nonpathogenic strains of *C. sordellii* was intermediate between the other two.

Since the discovery of *Clostridium bifermentans* by Tissier and Martelly (1902) and that of *C. sordellii* by Sordelli (1922), the taxonomic relationship of the two species has been the subject of controversy. Recently, Brooks and Epps (1959) reviewed the topic and also attempted to elucidate the taxonomic relationship by using 60 strains assigned to either group. They demonstrated that, in addition to two main differentials, such as toxigenicity and urease activity (Tataki and Huet, 1953), seven biological criteria for distinction between the two species could be found; in view of this, they insisted that the present practice of some bacteriologists of combining the two species under the same name *C. bifermentans* is incorrect and should be discontinued.

Huang (1959) disagreed with this view. He obtained nonpathogenic single-cell isolates from a toxigenic parent strain and demonstrated that these isolates were culturally, biochemically, and serologically indistinguishable from strains of *C. bifermentans*. He concluded, therefore, that *C. bifermentans* is a nonpathogenic strain of *C. sordellii*.

Since 1961, we have engaged in the study of the taxonomic problem from a viewpoint which, to date, has not been adopted by others. This new aspect was suggested by the findings of Yamagishi, Ishida, and Nishida (1964) that pathogenic and nonpathogenic strains of *C. perfringens* were significantly different in sporulating potency and also that inability to sporulate was associated with toxigenesis. When we found that strains of *C. bifermentans* sporulated to a greater degree than did strains of *C. sordellii*, we undertook a study of the factors concerned, using the approach described by Yamagishi et al. (1964).

### Materials and Methods

*Strains used.* All strains of *C. bifermentans* used were isolated in our laboratory. They were identified by the characteristics described by Sterne and van Heyningen (1958) and by Smith (1955). In later experiments, however, the method of Willis and Hobbs (1958) was found to be more convenient.

*C. sordellii* strains 3703, 1732, 4709, 4708, 4707, 1694, and 1733 were kindly provided by I. Batty, Wellcome Research Laboratories, Beckenham, England. *C. sordellii* strain 1734 was kindly provided by C. T. Huang, Hong Kong University.

Nonpathogenic *C. sordellii* strains 1619, 1620, 1621, 1623, and 6929 were also received from Wellcome Research Laboratories.

The properties of all nonpathogenic and pathogenic strains of *C. sordellii* were in agreement with those described by Brooks and Epps (1959).

*Subculture and stock culture medium.* Chopped-meat broth (10%, v/v) was used.

*Medium for toxin production.* Among the media

TABLE 1. *Toxigenicity and urease activity of heat-resistant substrains of Clostridium sordellii 1734*

| Subtrain | Toxigenicity ($LD_{50}$) | Urease activity |
|---|---|---|
| Control (unheated) | 875 | + |
| Substrain resistant to 70 C for 10 min | 875 | + |
| Substrain resistant to 80 C for 10 min | 25 | + |
| Substrain resistant to 90 C for 10 min | 0 | − |

tested, such as the digest broth of Pope and Smith (1932) and 10 or 20% chopped-meat broth, the last, adjusted to pH 7.0, was found to give the highest yield of lethal toxin. The peptone concentration was 3%, and the optimal duration of incubation was 48 hr.

*Estimation of toxicity.* The 48-hr culture was centrifuged, and the supernatant fluid was shaken several times a day with toluene. The supernatant fluid, when freed from toluene, was injected intravenously into mice. The toxin was usually determined as minimal lethal dose (MLD), with two mice per group; when more exact values were needed, the toxin was determined as $LD_{50}$, with five mice per group.

*Heat-resistance test.* It was found to be necessary to use different peptones for media for routine cultures and for heat-resistance tests. Poli peptone (Daigo Co., Osaka, Japan) was used for the former, and Arei peptone (Kyoei Co., Tokyo, Japan) for the latter.

To obtain heat-resistant substrains, heat selection was carried out on each strain by growing it in 10% chopped-meat broth for 24 hr and then distributing the culture into several small tubes (12 to 13 by 100 mm). Each portion was then heated at 80, 90, or 100 C for 10 or 20 min. Approximately 0.5 ml of each heated portion was transferred into fresh chopped-meat broth containing 1% glucose and incubated for 48 hr before examination.

The quantitative heat-resistance test was performed by determining the ratio of cells resistant to 90 C for 10 min to the total number of viable cells in 24-hr cultures. This definition will be covered in more detail in the Results. Difficulty was sometimes experienced in reading for growth in chopped-meat broth containing 1% glucose, but the addition of a drop of pH indicator (methyl red, 0.1 g; bromothymol blue, 0.2 g, 95% alcohol, 300 ml, and distilled water, 200 ml) was helpful.

## RESULTS

*Selection of C. bifermentans-like strains from C. sordellii 1734.* A preliminary experiment was performed with a strain of *C. sordellii* (1734) to determine the relationship among toxigenicity, urease activity, and sporulating potency. From this strain, substrains with different degrees of heat resistance were obtained by heating portions of the 48-hr chopped-meat broth culture at various temperatures. Four portions were heated at 70, 80, 90, and 100 C for 10 min, and the remaining portion was used as the control. The portion heated at 100 C did not contain any surviving cells.

The heat-resistant substrains were examined for toxigenicity and urease activity. When higher temperatures were used, fewer toxigenic strains were obtained (Table 1). The substrain resistant to 90 C for 10 min lost both toxigenicity and urease activity. This strain was re-examined 2 months later and was still nontoxigenic. No distinction could be demonstrated between the prototrophic and the heat-resistant strains except the two differences mentioned above and some increase of proteolytic activity in the heat-resistant strain.

Because the heat-resistant strain could not be clearly differentiated from *C. bifermentans*, an accidental contamination of the culture of *C. sordellii* by *C. bifermentans* was suspected. To check this hypothesis, *C. sordellii* 1734 was streaked on Zeissler's agar, and 20 substrains were obtained from isolated colonies. These substrains were examined for toxigenicity and urease activity. All strains were urease-positive, with toxigenicity ranging between 510 and 910 $LD_{50}$ (Table 2). These 20 substrains were cultivated for 48 hr, and about 1 ml of each in small test tubes was heated at 90 C for 10 min. All 20 cultures were found to contain heat-resistant organisms. The urease activity and toxigenicity of these heat-resistant substrains were examined and, as illustrated in Table 2, were demonstrated to be negative. This finding indicates that contamination could not be responsible for the phenomenon encountered in the above experiment.

*Selection of substrains similar to C. bifermentans from strains of C. sordellii by heat selection.* Further experiments were undertaken with other strains of *C. sordellii*. Thirteen pathogenic and nonpathogenic strains of *C. sordellii* were tested for heat resistance at 80, 90, and 100 C for 10 min and at 100 C for 20 min. A single substrain resistant to the highest heating temperature was obtained from each strain for the following determination of toxigenicity and urease activity.

All prototrophic strains of *C. bifermentans* were nontoxigenic and urease-negative, all prototrophic strains of *C. sordellii* N-P group were nontoxigenic and urease-positive, and all prototrophic strains of *C. sordellii* were urease-positive and toxigenic, although they varied a good deal in levels of toxigenicity. Among the heat-resistant substrains of the eight pathogenic strains used, seven were found to be nontoxigenic and the remaining strain was only slightly toxigenic (5 MLD/ml). Urease production, however, did not display such marked variation. Among 13 heat-resistant substrains of *C. sordellii* and *C. sordellii* N-P group, 6 were urease-negative and 7 were urease-positive. These findings indicate that strains similar to *C. bifermentans* can be obtained from pathogenic and nonpathogenic strains of *C. sordellii* and that strains similar to *C. sordellii* N-P group can be obtained from the pathogenic strains of *C. sordellii*.

TABLE 2. *Toxigenicity and urease activity of 20 substrains* of Clostridium sordellii 1734 and of their heat-resistant substrains†*

| Substrain no. | Parent strains | | Heat-resistant progenies | |
|---|---|---|---|---|
| | Toxigenicity (MLD) | Urease activity | Toxigenicity (MLD) | Urease activity |
| 1 | 800 | + | 0 | − |
| 2 | 800 | + | 0 | − |
| 3 | 400 | + | 0 | − |
| 4 | 800 | + | 0 | − |
| 5 | 800 | + | 0 | − |
| 6 | 400 | + | 0 | − |
| 7 | 400 | + | 0 | − |
| 8 | 800 | + | 0 | − |
| 9 | 800 | + | 0 | − |
| 10 | 800 | + | 0 | − |
| 11 | 800 | + | 0 | − |
| 12 | 400 | + | 0 | − |
| 13 | 800 | + | 0 | − |
| 14 | 400 | + | 0 | − |
| 15 | 800 | + | 0 | − |
| 16 | 400 | + | 0 | − |
| 17 | 800 | + | 0 | − |
| 18 | 400 | + | 0 | − |
| 19 | 400 | + | 0 | − |
| 20 | 800 | + | 0 | − |

* The 20 substrains were obtained from 20 colonies grown on Zeissler's plate culture.

† Heat-selection was performed at 90 C for 10 min.

TABLE 3. *Toxigenicity, urease activity, and heat-resistant nature of Clostridium sordellii, C. sordellii N-P group, C. bifermentans, and their heat-resistant progenies*

| Organism | Strain | Parent strains | | Temp used* | Heat-resistant substrains | |
|---|---|---|---|---|---|---|
| | | Urease activity | Toxigenicity (MLD) | | Urease activity | Toxigenicity (MLD) |
| | | | | C | | |
| C. bifermentans | 317 | − | 0 | 90 | | |
| | 315 | − | 0 | 90 | | |
| | 181 | − | 0 | 100 | | |
| | 212 | − | 0 | 100 | | |
| | 7033 | − | 0 | 100 | | |
| | 7034 | − | 0 | 90 | | |
| | 7036 | − | 0 | 90 | | |
| | 7037 | − | 0 | 90 | | |
| | 7038 | − | 0 | 90 | | |
| | 7031 | − | 0 | 100 | | |
| | 302 | − | 0 | 90 | | |
| | 319 | − | 0 | 90 | | |
| C. sordellii N-P group | 1619 | + | 0 | 100 | − | 0 |
| | 1620 | + | 0 | 100 | − | 0 |
| | 1621 | + | 0 | 100 | + | 0 |
| | 1623 | + | 0 | 90 | + | 0 |
| | 6929 | + | 0 | 100 | + | 0 |
| C. sordellii | 4707 | + | 200 | 100 | − | 0 |
| | 4708 | + | 200 | 90 | − | 0 |
| | 4709 | + | 400 | 100 | + | 0 |
| | 1732 | + | 400 | 90 | + | 0 |
| | 1733 | + | 100 | 90 | − | 0 |
| | 1734 | + | 800 | 90 | − | 0 |
| | 3703 | + | 1,200 | 100 | + | 5 |
| | 1694 | + | 100 | 90 | + | 0 |

* The maximal heating temperature which each strain could resist for 10 min.

*Heat-resistant nature of C. sordellii and C. bifermentans.* The data in Table 3 indicate no difference in the heat resistance of the two species

TABLE 4. *Sporulating potency of Clostridium sordellii 1734, C. bifermentans 317, and their substrains*

| Strain | *C. sordellii* 1734 | | *C. bifermentans* 317 | |
|---|---|---|---|---|
| | No. of live cells (per ml) | Cells resistant to 90 C for 10 min (per ml) | No. of live cells (per ml) | Cells resistant to 90 C for 10 min (per ml) |
| Parent strain | $3.2 \times 10^8$ | $1.3 \times 10^2$ | $4.6 \times 10^8$ | $3.3 \times 10^8$ |
| Substrain no. | | | | |
| 1 | $1.7 \times 10^8$ | $1.7 \times 10^2$ | $2.6 \times 10^8$ | $1.7 \times 10^8$ |
| 2 | $2.6 \times 10^8$ | $4.0 \times 10^1$ | $3.3 \times 10^8$ | $2.6 \times 10^8$ |
| 3 | $1.1 \times 10^8$ | $7.8 \times 10^1$ | $1.1 \times 10^8$ | $9.3 \times 10^7$ |
| 4 | $1.1 \times 10^7$ | $1\text{–}10^1$ | $1.4 \times 10^8$ | $1.1 \times 10^8$ |
| 5 | $5.4 \times 10^8$ | $1\text{–}10^1$ | $9.3 \times 10^8$ | $1.1 \times 10^8$ |
| 6 | $2.2 \times 10^7$ | $1\text{–}10^1$ | $1.4 \times 10^8$ | $6.8 \times 10^7$ |
| 7 | $2.6 \times 10^8$ | $4.0 \times 10^1$ | $4.0 \times 10^8$ | $1.7 \times 10^8$ |
| 8 | $1.4 \times 10^8$ | $6.8 \times 10^2$ | $2.0 \times 10^7$ | $2.0 \times 10^7$ |
| 9 | $2.2 \times 10^7$ | $1.3 \times 10^2$ | $4.0 \times 10^7$ | $4.0 \times 10^7$ |
| 10 | $2.2 \times 10^8$ | $2.2 \times 10^2$ | $2.0 \times 10^8$ | $1.4 \times 10^8$ |

with the test employed. Because this finding was in complete agreement with the finding that substrains similar to *C. bifermentans* could be obtained from *C. sordellii* by heat selection, further experiments were performed to determine their heat-resistant nature by the method described by Yamagishi et al. (1964).

Cultures of *C. sordellii* 1734, *C. sordellii* N-P 6929, and *C. bifermentans* 317 were streaked on Zeissler's agar, and 50 colonies from each strain were transferred into chopped-meat broth. Each substrain thus established was subjected to the heat-resistance test at 90 C for 10 min as described previously. The number of tubes showing growth was determined after 48 hr of incubation. Growth was evident in 48 of the 50 tubes of *C. sordellii* 1734, and in all tubes of *C. sordellii* N-P and *C. bifermentans*. Thus, no distinction could be made between these species under these conditions.

The classification "heat-resistant" seems to be ambiguous because, although it is a qualitative term, it must be measured and interpreted quantitatively (Yamagishi et al., 1964). This was done by determining the ratio of the number of cells surviving 90 C for 10 min to the total number of viable cells in a 24-hr culture in chopped-meat broth. Because the heat resistance is related to sporulation, this ratio is referred to as "sporulating potency." This has already been used by Yamagishi et al. (1964) in a study of heat resistance in *C. perfringens*. For the present study, *C. sordellii* 1734 and *C. bifermentans* 317 were employed. *C. sordellii* 1734 had $1.3 \times 10^2$ heat-resistant cells in a total of $3.2 \times 10^8$ live cells per ml, and *C. bifermentans* 317 had $3.3 \times 10^8$ heat-resistant cells in a total of $4.6 \times 10^8$ live cells per ml (Table 4). Both strains were streaked again on Zeissler's agar, and ten substrains were established from ten colonies of each strain. These substrains were subjected to the quantitative heat-resistance test. The ten substrains of *C. sordellii* were shown to possess sporulating potency approximately equal to that of the prototrophic strain; the ten substrains of *C. bifermentans* also possessed sporulating potency similar to that of their prototrophic strain. The same type of experiment was repeated with four other strains possessing various sporulating potencies, and consistent results were always obtained. These results indicate that a strain exhibiting a certain level of sporulating potency consists mainly of individual cells possessing a similar sporulating potency. In other words, the sporulating potency of $10^2$ heat-resistant cells in a total of $10^8$ cells does not mean that the strain consists of $10^2$ cells with potential sporulating ability and approximately $10^8(10^8 - 10^2)$ cells with no potential sporulating ability, but means that the strain consists mainly of individual cells possessing sporulating ability similar to that of the prototroph. Since the sporulating potency of the same strain varied considerably in the different media used and sometimes was even influenced by differences in batches of the same medium

prepared on different dates, the sporulating potency should be regarded as a phenotypic expression of the sporulating ability of the strain under the particular conditions used.

The above experiment disclosed that there were remarkable differences in sporulating potency between *C. sordellii* 1734 and *C. bifermentans* 317. Therefore, to compare the sporulating potency of other strains, five strains of *C. bifermentans*, five strains of *C. sordellii* N-P group, and seven strains of *C. sordellii* were tested. The sporulating potency ratio of all strains of *C. sordellii* ranged between $10^0$ and $10^2$ resistant cells in a total of $10^7$ to $10^8$ cells; *C. bifermentans* had a ratio of $10^7$ to $10^8$ resistant cells in a total of $10^7$ to $10^8$ cells; and *C. sordellii* N-P group had a ratio of $10^5$ to $10^6$ resistant cells in a total of $10^7$ to $10^8$ cells per ml (Table 5). These findings demonstrate that *C. sordellii*, *C. sordellii* N-P group, and *C. bifermentans* possess distinctive sporulating potencies.

TABLE 5. *Sporulating potency of Clostridium sordellii, C. sordellii N-P group, and C. bifermentans*

| Strains used | No. of live cells per ml | Cells resistant to 90 C for 10 min per ml |
|---|---|---|
| *C. bifermentans* | | |
| 315 | $3.3 \times 10^7$ | $1.1 \times 10^7$ |
| 181 | $2.7 \times 10^8$ | $2.3 \times 10^8$ |
| 317 | $1.1 \times 10^8$ | $6.8 \times 10^7$ |
| 302 | $4.9 \times 10^8$ | $1.4 \times 10^8$ |
| 319 | $3.3 \times 10^7$ | $1.4 \times 10^7$ |
| *C. sordellii N-P group* | | |
| 1619 | $4.0 \times 10^8$ | $1.4 \times 10^5$ |
| 1620 | $1.1 \times 10^8$ | $2.0 \times 10^6$ |
| 1621 | $6.8 \times 10^8$ | $7.8 \times 10^6$ |
| 1623 | $1.1 \times 10^8$ | $2.0 \times 10^5$ |
| 6929 | $6.8 \times 10^8$ | $2.0 \times 10^5$ |
| *C. sordellii* | | |
| 1733 | $1.7 \times 10^8$ | $1-10^1$ |
| 4707 | $7.9 \times 10^8$ | $4.5 \times 10^1$ |
| 4708 | $6.8 \times 10^7$ | $1-10^1$ |
| 1732 | $2.2 \times 10^8$ | $1.7 \times 10^2$ |
| 3703 | $3.3 \times 10^8$ | $1.7 \times 10^2$ |
| 4709 | $1.4 \times 10^8$ | $2.0 \times 10^1$ |
| 1694 | $4.9 \times 10^8$ | $1-10^1$ |

## DISCUSSION

Several workers have described unexpected findings during the course of taxonomic studies on *C. bifermentans* and *C. sordellii*. It seems to be generally accepted that Sordelli's pathogenic isolate "82" is liable to produce nonpathogenic mutants. We have had similar experiences with strain "82," which we received from C. T. Huang, Hong Kong University, early in 1960. Immediately on receipt, the biological characteristics, toxigenicity, and urease activity were examined. However, it was demonstrated that the strain was urease-negative and nontoxigenic and, consequently, quite similar to *C. bifermentans* except for colonial morphology. This strain is now in stock culture in our laboratory, and occasional examinations have always shown it to be nontoxigenic and urease-negative.

In 1963, we were provided with another strain of "82" by A. R. Prévot, Pasteur Institute, Paris, France. An immediate examination showed that the strain was toxigenic and urease-positive. However, a nontoxigenic and urease-positive substrain could be obtained from it by selection for heat resistance.

After a thorough investigation of both species, Brooks and Epps (1959) reported that many strains of *C. bifermentans* suppressed the urease activity of *C. sordellii* when they were mixed with it. It is suggested, in the light of the investigations mentioned above, that these findings may be due to the stronger survival ability of *C. bifermentans* than *C. sordellii*. The role of sporulating potency in the taxonomy of both species will be reinvestigated with reference to many differentials established by Brooks and Epps and will be published in forthcoming papers. Serological analysis is also left for future investigation.

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