REINVESTIGATION OF THE TAXONOMY OF *CLOSTRIDIUM BIFERMENTANS* AND *CLOSTRIDIUM SORDELLII*

S. NAKAMURA, T. SHIMAMURA, H. HAYASHI AND S. NISHIDA

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

THE taxonomic relationship between *Clostridium bifermentans* and *C. sordellii* has been well reviewed by Brooks and Epps (1958) and was subsequently studied by Nishida, Tamai and Yamagishi (1964). In view of Novotný's recent claim (1969) that both species should be classified on the basis of sugar components in the cell walls, and because of the similarity of both species in their cultural and biochemical properties, the problem was reinvestigated in the present study by means of numerical taxonomy and consideration of DNA-DNA homology, thermostability of homologous and heterologous duplexes, and analysis of sugar components in the cell walls.

MATERIALS AND METHODS

Strains used. The sources and designations of the strains are shown in table I.

C. sordellii strain 82 was obtained from the Department of Bacteriology, Leeds University, in 1959; at that time, the strain was non-toxigenic and urease negative. The subsequent subculture of this strain stocked in cooked-meat broth is designated as strain 82-Kanazawa (82-KZ) in the present text. Strains 82-SJ2, 82-SJ3, and 82-SJ4 are the substrains of *C. sordellii* strain 82 stocked in Leeds University by single-cell isolation (Huang, 1959). A culture of *C. sordellii* strain 82 was also received from Dr A. R. Prévot, Pasteur Institute, Paris, in 1963; this strain is still toxigenic and urease positive and is designated as *C. sordellii* no. 82-Pasteur (82-P). *C. sordellii* no. 7222R/100 is a non-toxigenic substrain obtained by heating the toxigenic *C. sordellii* strain 7222R at 100°C for 30 min. *C. sordellii* no. 1734/90 is another non-toxigenic substrain obtained by heating a toxigenic strain 1734 at 90°C for 30 min. (Huang, Tamai and Nishida, 1965).

Numerical taxonomy. The properties used in the characterisation of the organisms were previously described by Nakamura *et al.* (1973). The substrates used for tests of deaminase activity are alanine, aspartate, arginine, glutamate, leucine, methionine, phenylalanine, serine, threonine, and valine. The number of features scored is 162. Procedures for the determination of the strain characteristics, similarity value (S-value), coding of data, and analysis of the S-value have been described previously (Nakamura *et al.*, 1973).

Determination of sugar components of cell walls. Paper chromatography with Toyo filter-paper no. 51 was used according to the method described by Cummins and Johnson (1971). The bacterial cells were cultured in PY medium containing 1% fructose (PYF medium). The PY medium consisted (w/v) of proteose peptone (Difco No. 2) 2%, yeast extract (Daigo, Tokyo, Japan) 0.5%, NaCl 0.5%, and sodium thioglycollate 0.1%. The culture was harvested usually in the late logarithmic phase.

Growth inhibition by mannose. Volumes (0.1 ml) of 12-h cultures grown in the PY medium with 0.5% fructose were transferred into 10-ml volumes of the PY medium with or without 1% mannose and incubated at 37°C. Bacterial growth was measured for optical density at

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TABLE I

Sources and designations of organisms employed in numerical taxonomy and DNA reassociation studies

Name and strain number, as designated when isolated or received	Source or reference
<i>C. bifermentans</i> 181, 317, 302	Strains isolated from soils, Kanazawa, Japan (Nishida, Tamai and Yamagishi, 1964)
7031, 7033, 7034, 7036, 7037, 7039 —> 506	Strains isolated from hen faeces, Kanazawa, Japan (Nishida <i>et al.</i> , 1964)
D-6	Huang, Hong Kong University, Hong Kong (originally NCTC 506) National Institute of Health, Tokyo, Japan
<i>C. bifermentans</i> -like strains 82-KZ, 82-SJ2, 82-SJ3, 6927 7222R/100	Huang, Hong Kong (Huang, 1959) Huang, Tamai and Nishida (1965)
<i>C. sordellii</i> 1732, 1619, 1620, 1621, 1623, 3703, 4707, 4708, 4709 6929, 6800, 1340, 1733, 1734, 7222R, 82-SJ4 1734/90 82-P	Mrs Irene Batty, Wellcome Research Laboratories, Beckenham, England Huang, Hong Kong (Huang, 1959) Huang <i>et al.</i> (1965) Prévot, Pasteur Institute, Paris
<i>C. sporogenes</i> 173, 14	Strains isolated from soil, Kanazawa, Japan
<i>C. botulinum</i> type A: 97-S type A: 190, type E: Iwanai, Tenno	National Institute of Health, Tokyo, Japan Hokkaido Institute of Public Health, Hokkaido, Japan

560 nm (E_{560}) with a Shimazu-Bosch-Lomb Spectronic 20 spectrophotometer (Shimazu Co. Kyoto, Japan) after incubation for 12, 24, 48, and 72 h. The pH values of cultures were measured with a Zeromatic SS-3 pH meter (Beckman-Toshiba Ltd, Tokyo, Japan) at the final harvest time.

Preparation of DNA. The cells were grown in the PYF medium. For preparation of ^3H -labelled DNA, 10 μCi of ^3H -thymidine was added to 100 ml of PYF medium without yeast extract. Penicillin G was added to a concentration of 50 μg per ml in the late logarithmic phase. The culture was incubated for 30 min. at 37°C with further incubation for 10 min. at 60°C. The treated cells were then harvested by centrifugation, and suspended in 0.15M-NaCl solution containing 0.01M-ethylenediamine tetraacetic acid (EDTA) at pH 8.0. The cell preparation was stored at -25°C and thawed when used. The cell suspension was treated with lysozyme 1 mg per ml and lysed with 3% sodium lauryl sulphate (SLS). Extraction of DNA from this lysate was performed according to the method of Marmur (1961).

DNA homology studies. The competition test with membrane filter was performed according to the method described by Johnson and Ordal (1968) and Johnson and Cummins (1972). Ten μl of labelled DNA fragments (1.0 μg DNA) homologous to the DNA on the filter (reference DNA) and 100 μl of 2.2-strength salt-sodium citrate (SSC) solution (SSC consists of 0.15M-NaCl and 0.015M-sodium citrate at pH 7.0) or competitor DNA (75 μg usually and

FIG. 1.—Dendrogram of *C. bifermentans*, *C. sordellii*, and reference strains. *B = *C. bifermentans*; BL = *C. bifermentans*-like strain; S = toxigenic *C. sordellii*; NS = non-toxigenic *C. sordellii*.

TABLE II

Mean similarities of each phenon-II strain (*C. sordellii*) to other strains in the same phenon (II) and to all strains in another phenon (I)

Strain in phenon II	Mean similarity value (%) to	
	phenon I	phenon II
<i>C. sordellii</i>		
1734	84	86
1620	83	90
1621	83	89
6800	83	89
3703	81	89
1733	81	90
6929	81	92
7222R	80	92
1732	80	89
1623	80	91
4709	80	89
1340	79	87
4707	79	90
82-SJ4	78	89
4708	78	90
82-P	77	88

150 µg occasionally) in 2.2-strength SSC were added to incubation vials (6 by 25 mm). The size of the small filter was 3 × 9 mm. The vials were incubated for 15 h at 60°C. After incubation, filters were washed in double-strength SSC at the temperature used for incubation, dried, and counted in a liquid scintillation system, Unilex I-A (Nuclear-Chicago Co., Des Plaines, Ill., USA).

Investigation of the thermostability of DNA duplexes. Homologous or heterologous DNA duplexes on filters were formed by the above-mentioned procedure. The filters washed in double-strength SSC were transfixed with an insect pin and placed in a Wassermann tube (13 × 100 mm) containing 1.2 ml of 0.5-strength SSC; the tube was then placed in a water bath. The filters were transferred at 10-min. intervals through a series of tubes with 5°C temperature increments at each interval. The water bath reached each new temperature in approximately 5 min. Each tube was emptied into a scintillation vial and washed in with 0.5 ml of 0.5-strength SSC, and then 10 ml of a preparation of Triton X-100 counting solution (3 parts toluene counting solution and 2 parts Triton X-100) was added to each vial which was finally transferred to a liquid scintillation counter.

TABLE III

Some biochemical criteria differentiating phenon I from phenon II

Phenon	Number of strains included	Number of strains giving a positive result in tests for								
		fermentation of		growth inhibition by mannose*	phosphatase	urease	deaminase for			toxi- genicity
		mannose	sorbitol				arginine	valine	alanine	
I	14	12*	13	0	8	0	14	7	8	0
II	16	1	1	15	0	16	1	0	0	9

* See Methods and table IV.

RESULTS

Eighteen toxigenic and non-toxigenic strains of *C. sordellii*, 11 strains of *C. bifermentans* and 5 *C. bifermentans*-like strains that are labelled as *C. sordellii* but are non-toxigenic and urease negative were used for this study. As presented in fig. 1, all of these strains are linked at a high S-value (88%) and associated at a low S-value (82%) with reference strains of *C. sporogenes* and *C. botulinum* type A. The strains in the 88-phenon were sorted into two groups, phenons I and II at an S-value of 91%. Phenon I included *C. bifermentans* and the *C. bifermentans*-like strains, whilst phenon II incorporated

TABLE IV
Inhibition of growth of certain organisms by mannose

Organism	Optical density (E_{560}) of culture at 24 h in medium*		pH value after culture for 72 h in medium*	
	PY	PYM	PY	PYM
Phenon I				
506 (B)†	0.48	1.24	8.07	5.52
D-6 (B)	0.34	0.58	7.84	6.52
7033 (B)	0.26	0.96	8.10	5.43
7036 (B)	0.30	0.96	8.02	5.30
7031 (B)	0.40	0.80	8.70	5.47
7034 (B)	0.33	0.89	8.07	5.34
7039 (B)	0.37	1.05	8.00	5.38
82-KZ (BL)	0.57	1.30	8.10	5.70
82-SJ3 (BL)	0.47	1.22	7.98	5.58
317 (B)	0.34	1.18	8.12	5.54
6929 (BL)	0.55	1.16	8.09	5.61
7037 (B)	0.40	0.90	8.00	5.42
302 (B)	0.54	1.05	8.04	6.55
82-SJ2 (BL)	0.47	1.10	7.87	5.95
Phenon II				
1620 (NS)	0.44	0.05	8.00	7.60
6800 (NS)	0.42	0.25	7.99	7.71
1733 (S)	0.29	0.30	7.92	7.57
1734 (S)	0.21	0.10	7.90	7.82
4708 (S)	0.32	0.10	7.94	7.60
7222R (S)	0.28	0.15	8.05	7.81
6929 (NS)	0.69	0.50	8.00	7.74
4707 (S)	0.31	0.05	7.86	7.44
1340 (NS)	0.54	0.22	7.90	7.70
1623 (NS)	0.41	0.09	8.00	7.64
1621 (NS)	0.49	0.23	8.05	7.70
3703 (S)	0.48	0.31	8.04	7.57
1732 (S)	0.31	0.01	8.02	7.40
82-SJ4 (S)	0.49	0.04	8.02	7.56
4709 (NS)	0.53	0.10	8.02	7.38
82-P (S)	0.29	0.05	8.04	7.50
90-phenon				
7222R/100 (BL)	0.62	1.15	8.05	5.53
1619 (NS)	0.47	0.07	8.00	7.58
1734/90 (NS)	0.37	0.38	8.10	7.78

* PY = peptone yeast-extract medium; PYM = PY medium with mannose (1%) (see *Methods*).

† B = *C. bifermentans*; BL = *C. bifermentans*-like strain; S = toxigenic *C. sordellii*; NS = non-toxigenic *C. sordellii*.

the toxigenic and non-toxigenic strains of *C. sordellii*. In table II, the mean S-value of each strain of phenon II for all other strains in the same phenon is compared with its mean S-value for all strains in phenon I. Although the results suggest that both phenons are extremely close to each other, characteristics that differentiate the strains in phenon I from the strains in phenon II were fermentation of mannose and sorbitol, urease activity, arginine deamination, and growth inhibition by mannose (table III). Mannose enhanced the growth of strains in phenon I, but inhibited the growth of strains in phenon II when present at a concentration of 1% in PY culture medium. The results

TABLE V
Cell-wall sugars of *Clostridium sordellii* and *C. bifermentans*

Strain number	Cell-wall sugars present (+) or absent (-)*			
	galactose	glucose	mannose	rhamnose
<i>C. sordellii</i>				
1733	+	+	-	-
1734	-	++	-	-
4707	-	tr	-	-
4708	-	+	-	-
3703	+	+	-	-
82-P	tr	++	-	-
82-SJ4	-	++	-	-
1623	+	-	-	-
1340	tr	tr	-	-
1619	-	+	-	-
1620	-	++	-	-
6800	tr	tr	-	-
6929	+	+	-	-
1621	tr	++	-	-
4709	tr	tr	-	-
7222R	-	++	-	-
1732	-	++	-	-
1734/90	tr	++	-	-
<i>C. bifermentans</i>				
302	-	++	++	++
506	-	+	+	+
317	-	+	++	-
181	+	+	+	+
7031	+	++	++	-
7033	+	+	+	-
7034	+	+	tr	-
7036	+	+	+	-
7037	+	+	+	-
7039	+	++	+	-
D-6	+	tr	+	++
<i>C. bifermentans</i> - like strains				
82-KZ	++	++	-	-
82-SJ2	-	+	-	-
82-SJ3	-	++	++	+
7222/100	-	++	++	++
6927	-	++	++	++

* tr = Trace; +, ++ indicate the relative amounts present.

are given in detail in table IV. Since maximal growth was reached at 12–24 h and lysis occurred thereafter, only the results at 24 h are shown in the table. Of the other 16 sugars tested, none showed a growth-inhibitory effect.

Table V summarises the results of our studies on the sugar components of cell walls of *C. sordellii* and *C. bifermentans*. Glucose was demonstrated in all but one of the strains examined. Neither mannose nor rhamnose was present in any strain of *C. sordellii*, whereas mannose was demonstrated in all strains of *C. bifermentans* tested and rhamnose was present in some. Five strains of *C. bifermentans*, viz., nos. 7031, 7033, 7034, 7037 and 7039, which consistently produced extraordinarily minute colonies with wide haemolytic zones on blood agar, had cell walls with glucose, mannose, and galactose but without rhamnose. Glucose and galactose were notably present and mannose and rhamnose were absent from the cell walls of the non-toxicogenic, urease-negative strain 82-KZ, which originated from a toxicogenic *C. sordellii* strain no. 82; the toxicogenic *C. sordellii* strain 82-P, possibly of the same origin as strain 82-KZ, showed glucose and a trace of galactose in its cell wall. Strains 82-SJ2, 82-SJ3 and 82-SJ4 are single-cell isolates derived from toxi-

TABLE VI
Polynucleotide sequence relationships between strains of phenons I and II

Organism	Data obtained in comparative tests with			
	strain 1620 (phenon II)		strain 82-SJ2 (phenon I)	
	Percentage homology	$\Delta T_m(e)$	Percentage homology	$\Delta T_m(e)$
Phenon II				
1620 (S)*	100	0	51	9.5
1734 (S)	100	...†	52	...
82-SJ4 (S)	99	0	72	9.9
1621 (NS)	97	...	72	...
4708 (NS)	97	...	71	...
1732 (S)	96	0.3	72	9.9
6800 (NS)	95	0	69	10.5
7222R (S)	94	...	69	...
3703 (S)	92	0.4	43	9.9
82-P (S)	88	...	55	...
Phenon I				
82-KZ (BL)	71	...	91	...
82-SJ3 (BL)	63	9.8	100	0.2
7034 (B)	62	9.7	97	0.5
7037 (B)	60	...	99	...
7033 (B)	57	...	100	...
7039 (B)	54	...	100	...
317 (B)	51	7.8	92	2.6
82-SJ2 (BL)	46	8.8	100	0
90-Phenon				
1734/90 (NS)	99	...	69	...
7222R/100 (BL)	60	...	98	...

* See footnote to table IV.

† ... = Not tested.

genic strain 82 but varying in their toxigenicity and urease activity; they also vary in their cell-wall sugar components. Glucose alone was found in strain 82-SJ2 which is non-toxigenic and urease-negative, and glucose alone was found in strain 82-SJ4 which is toxigenic and urease positive. Strain 82-SJ3 shows the typical properties of *C. bifermentans*; it is non-toxigenic, urease negative and has glucose, mannose and rhamnose in its cell wall.

The relationship between phenons I and II was further studied with DNA-DNA homology tests. Although strain 82-SJ2 in phenon I originated from *C. sordellii* strain 82, strain 82-SJ2 was used as the reference strain of phenon I because it exhibited the typical properties of *C. bifermentans* in cultural and biochemical tests and yet was linked by a high mean S value of 90% with strains in phenon II. The degree of DNA homology between strain 82-SJ2 of phenon I and each strain of phenon II ranged between 43 and 72%, whilst

FIG. 2.—Thermo-stability profiles of homologous and heterologous duplexes of a strain 82-SJ2 of phenon I and a strain 1620 of phenon II. Strain 82-SJ2, ^3H -DNA: $\triangle$ homologous, $\blacktriangle$ heterologous; strain 1620, ^3H -DNA: $\circ$ homologous, $\bullet$ heterologous. The reassociations were performed in double-strength SSC at 60°C. The elution profiles were performed in half-strength SSC.

the homology between strain 82-SJ2 and each strain of the same phenon ranged between 91 and 100% (table VI). Similar results were obtained when homology of the reference strain 1620 of phenon II was assessed in relation to the strains of phenons I and II. These findings indicate that although strains of both phenons are intimately related they may still be differentiated.

Thermal stability tests were performed on the homologous DNA duplexes produced between strains of the same phenon and on the heterologous DNA duplexes formed between strains in different phenons. Fig. 2 depicts the homologous and heterologous thermostability profiles of strains 82-SJ2 in phenon I and strain 1620 in phenon II both of which were employed as reference strains in the DNA-DNA homology experiment. The temperature differences between homologous and heterologous duplexes at which 50% of the duplexes dissociated $\{T_m(e)\}$ are 9.5°C for strain 1620 which shows 51% homology with the strain 82-SJ2, and 8.8°C for strain 82-SJ2 which shows 46% homology with strain 1620. The results summarised in table VI reveal that, in spite of the fairly wide degree of variation in DNA-DNA homology, approximately the same levels of $\Delta T_m(e)$ could be obtained among strains of each phenon. The $\Delta T_m(e)$ of the heterologous DNA duplexes ranged from 7.8° to 10.5°C, whereas those of the homologous DNA duplexes were less than 2.6°C. This finding again indicates that DNA preparations of strains within each phenon are highly homologous and that a certain difference exists between the two phenons.

DISCUSSION

Johnson (1973) defined geno-species including geno-subspecies and genovarieties on the basis of DNA-DNA homology. Geno-subspecies were strains having 60 to 70% homology with organisms in the homologous group. Our present findings reveal that *C. bifermentans* and *C. sordellii* can be allocated into one geno-species, since phenon I consisting of *C. bifermentans* and *C. bifermentans*-like strains and phenon II consisting of *C. sordellii* had degrees of homology ranging from 43 to 71% with each other. Besides, the DNA hetero-duplexes formed between strains of phenons I and II showed $\Delta T_m(e)$ values ranging from 7.8 to 10.5°C. However, these two groups were differentiated clearly from each other by the tests for urease, arginine deaminase, fermentation of sorbitol and mannose, and growth inhibition by mannose. Novotný (1969), however, stated that two toxigenic strains of *C. sordellii* were urease negative. Also, fermentation of sugars is not a reliable criterion for differentiation when different culture media are employed (Tamai and Nishida, 1964). Novotný (1969) advocated a classification based on distinction of cell-wall sugars; e.g., *C. bifermentans* has a pattern of glucose, rhamnose and mannose or galactose; and *C. sordellii* has a pattern of glucose only or glucose and a trace of galactose. However, Rode and Smith (1971) indicated that two of six *C. bifermentans* strains tested had glucose only and the remainder had multiple combinations of glucose, rhamnose and mannose or galactose. The present study revealed varying sugar patterns with sub-strains of a toxigenic *C. sordellii* strain 82; strain 82-SJ2 in phenon I contained

glucose only, and strain 82-SJ3, also in phenon I, had glucose, mannose, and rhamnose, whereas strain 82-SJ4 in phenon II had glucose only.

Strain 82 has given rise to several non-toxicogenic substrains (Brooks and Epps, 1958). The cell wall of a non-toxicogenic, urease-negative strain 82-KZ contained glucose and galactose but no trace of rhamnose and mannose. The present results obtained with other strains disclosed that no definite pattern of cell-wall sugars could be found in either *C. bifermentans* or *C. sordellii*. In spite of the multiple sugar patterns, however, the present study demonstrates that the cell walls of both species were distinctly different in the mannose component, as stated by Novotný (1969); mannose was consistently absent from the cell-wall preparations of all 18 *C. sordellii* strains, whilst it was present in all of the preparations of the 11 *C. bifermentans* strains tested.

The manner whereby mannose inhibits the growth of *C. sordellii* but not *C. bifermentans* is not obvious. If there is a correlation between growth inhibition by mannose and the absence of mannose in the cell wall, it remains to be elucidated.

The present findings suggest that a clear distinction may not be found between *C. bifermentans* and *C. sordellii* when an increased number of strains are tested. This assumption seems reasonable in view of the results obtained in the DNA homology test. It should be noted that Huang, Tamai and Nishida (1965) obtained a *C. bifermentans*-like substrain by heating a culture of a toxigenic single-cell isolate 82-SJ4 derived from a reference strain of *C. sordellii*.

In spite of their close relatedness, however, we still consider it significant that *C. sordellii* should be differentiated from *C. bifermentans* on the basis of a few phenetic differences that were closely related to the small difference in the homology of their polynucleotide sequences, because toxigenic strains are included among *C. sordellii* strains but not among the *C. bifermentans* group.

Recently, the same kind of findings were made by Lee and Riemann (1970) and by Kiritani *et al.* (1973) in studies of *C. sporogenes* and *C. botulinum*.

SUMMARY

The taxonomic relationships between *Clostridium bifermentans* and *C. sordellii* were reinvestigated by numerical taxonomy, studies of DNA-DNA homology and DNA duplex thermal stability, and by analysis of cell-wall sugar components. Although the results indicate that both species may be grouped into one geno-species, *C. sordellii* strains could be differentiated from *C. bifermentans* strains on the basis of a few phenetic criteria that include the inability to ferment mannose and sorbitol, the absence of mannose in the cell wall, the production of urease, the absence of arginine deaminase activity, and susceptibility to inhibition of growth by mannose.

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REFERENCES

- BROOKS, M. E. AND EPPS, H. B. G. 1958. Taxonomic studies of the genus *Clostridium*: *Cl. bifermentans* and *Cl. sordellii*. *J. gen. Microbiol.*, **21**, 144.
- CUMMINS, C. S. AND JOHNSON, J. L. 1971. Taxonomy of the clostridia: wall composition and DNA homologies in *Clostridium butyricum* and other butyric acid-producing clostridia. *J. gen. Microbiol.*, **67**, 33.
- HUANG, C. T. 1959. Comparison of *Clostridium bifermentans* and *Clostridium sordellii*. Ph.D. Thesis, University of Leeds, Leeds, England.
- HUANG, C. T., TAMAI, K. AND NISHIDA, S. 1965. Taxonomy of *Clostridium bifermentans* and *Clostridium sordellii*. III. Agglutinability of heat-resistant substrain of *Clostridium sordellii*. *J. Bact.*, **90**, 391.
- JOHNSON, J. L. 1973. Use of nucleic-acid homologies in the taxonomy of anaerobic bacteria. *Int. J. syst. Bact.*, **23**, 308.
- JOHNSON, J. L. AND CUMMINS, C. S. 1972. Cell wall composition and deoxyribonucleic acid similarities among the anaerobic coryneform, classical propionibacteria, and strains of *Arachnia propionica*. *J. Bact.*, **109**, 1047.
- JOHNSON, J. L. AND ORDAL, E. J. 1968. Deoxyribonucleic acid homology in bacterial taxonomy: effect of incubation temperature on reaction specificity. *J. Bact.*, **95**, 893.
- KIRITANI, K., MITSUI, N., NAKAMURA, S. AND NISHIDA, S. 1973. Numerical taxonomy of *Clostridium botulinum* and *Clostridium sporogenes* strains and their susceptibilities to induced lysins and to mitomycin C. *Jap. J. Microbiol.*, **17**, 361.
- LEE, W. H. AND RIEMANN, H. 1970. The genetic relatedness of proteolytic *Clostridium botulinum* strains. *J. gen. Microbiol.*, **64**, 85.
- MARMUR, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. molec. Biol.*, **3**, 208.
- NAKAMURA, S., SHIMAMURA, T., HAYASE, M. AND NISHIDA, S. 1973. Numerical taxonomy of saccharolytic clostridia, particularly *Clostridium perfringens*-like strains; descriptions of *Clostridium absonum* sp. n. and *C. paraperfringens*. *Int. J. syst. Bact.*, **23**, 419.
- NISHIDA, S., TAMAI, K. AND YAMAGISHI, T. 1964. Taxonomy of *Clostridium bifermentans* and *Clostridium sordellii*. I. Their toxigenicity, urease activity and sporulating potency. *J. Bact.*, **88**, 1641.
- NOVOTONÝ, P. 1969. Composition of cell walls of *Clostridium sordellii* and *Clostridium bifermentans* and its relation to taxonomy. *J. med. Microbiol.*, **2**, 81.
- RODE, L. J. AND SMITH, L. DS. 1971. Taxonomic implications of spore fine structure in *Clostridium bifermentans*. *J. Bact.*, **105**, 349.
- TAMAI, K. AND NISHIDA, S. 1964. Taxonomy of *Clostridium bifermentans* and *Clostridium sordellii*. II. Toxigenic and sporulating potencies in substrains of *Clostridium sordellii* strain. *J. Bact.*, **88**, 1647.