THE APPLICATION OF BACTERIOPHAGE AND SEROLOGY IN THE DIFFERENTIATION OF STRAINS OF LEUCONOSTOC MESENTEROIDES

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The bacterial species *Leuconostoc mesenteroides* (Cienkowski) Van Tieghem as characterized by Bergey *et al.* (1939) includes four strains which are markedly different in colonial appearance when grown on sucrose agar. These strains, or types, have been designated A, B, D, and F by Faville (1947) and McCleskey, Faville, and Barnett (1947). These types also differ significantly in certain fermentative reactions and in the amount of gum produced. The purpose of the work reported in this paper was to determine whether or not these four types of *L. mesenteroides* comprise distinct entities as revealed by serological reactions and lysis by specific bacteriophages.

Some years ago Hucker (1932) attempted to utilize the agglutination reaction in the separation of species of the genera *Leuconostoc* and *Streptococcus* and concluded that these species "evidence a large amount of strain specificity." He found cross reactions between *Leuconostoc mesenteroides* and various streptococci to be common. Neill and associates (1941) have reported a serological relationship between the polysaccharides of *L. mesenteroides* and the type II pneumococcus.

In the serological studies both precipitation and agglutination methods were employed. For the precipitin tests antisera were prepared by injecting heat-killed cells intravenously into rabbits in accordance with the procedure of Lancefield (1933). The centrifuged bacterial sediment from 50 ml of a 24-hour culture grown in tryptone glucose yeast extract broth was resuspended in half volume of saline, and the cells were killed by heating at 56 C for 1 hour. One to 2 ml of this suspension were injected daily for 7 days, followed by a week's rest period. Three series of injections were given. On the fifth day after the last injection the rabbits were bled, and the separated sera were stored in the icebox until used.

The antigen extracts were prepared by a modification of the Lancefield technique. The bacterial sediment from 50 ml of a 24-hour broth culture was suspended in 2 ml of N/20 HCl in 0.85 per cent saline. The tube was immersed in a water bath at 60 C for 1 hour and then centrifuged. The supernatant liquid was removed and to it was added a drop of 0.04 per cent bromthymol blue. It was then neutralized with N/2 NaOH, centrifuged, and the sediment discarded. The remaining supernatant contained the antigen and was used in the precipitin test.

The tests were made in very small tubes, in which 0.05 ml of serum were placed and 0.05 ml of antigen layered over it. The tubes were examined for ring formation after 30 minutes at room temperature and again after 30 minutes at 37 C

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in the water bath. The contents of the tubes were then mixed, placed in the refrigerator, and observed the next day. All sera were controlled by testing with the homologous antigen and with a saline control.

In table 1 are presented the results of the precipitin tests using a total of 29 extracts. Of type A two extracts were tested against four sera; two were precipitated by 730(A) serum and none by sera 11(D), 548(D), and 860(F). Five type B extracts were tested of which 2 gave positive reactions with serum 730(A), 1 with serum 11(D), 2 with serum 548(D), and none with serum 860(F). In the D type 16 extracts were used, of which none was precipitated by serum 730, 2 by serum 11(D), 2 by serum 548(D), and none by serum 860(F). In the F type

TABLE 1

The precipitin test applied to the differentiation of Leuconostoc mesenteroides strains

TYPE	STRAINS TESTED	PER CENT POSITIVE PRECIPITATION REACTIONS			
		730(A)	11(D)	548(D)	860(F)
A	2	50	0	0	0
B	5	40	20	40	0
D	16	0	12.5	12.5	0
F	6	16.6	16.6	16.6	33.3

TABLE 2

Agglutination reactions in the Leuconostoc mesenteroides group

TYPE	STRAINS TESTED	PER CENT POSITIVE REACTIONS WITH ANTISERUM					
		730(A)	158(B)	11(D)	1060(D)	548(D)	860(F)
A	18	100*	0	0	0	0	5.5
B	30	0	6.6	0	0	0	0
D	50	22	10.5†	28	54	34	26
F	17	11.8	23‡	5.9	0	0	94.1

* Percentages are based on agglutination at 1:100 dilution.

† Thirty-eight strains tested.

‡ Thirteen strains tested.

we used 6 extracts, of which 2 were precipitated by serum 860(F) and 1 by each of the other sera (the same extract in each case).

For the agglutination tests the same sera were used. Four different dilutions were employed—1:50, 1:100, 1:200 or 1:400, and 1:600 or 1:1,000. The highest dilution employed was in each case the titer for the homologous organism. The tests were conducted in 0.5 per cent saline to reduce the likelihood of spontaneous agglutination. The antigens were 24- to 48-hour tryptone glucose yeast extract broth cultures.

Table 2 shows the results obtained in the 1:100 dilution or higher. Although there was cross agglutination between the four types, there was some evidence that certain types constitute reasonably distinct serological groups. Type A

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is apparently the most homogeneous, serologically, of the four types. The B strains were not agglutinated by any serum other than anti-B, but our single serum of that type agglutinated only one culture in addition to the homologous organism.

The results obtained in agglutinin absorption tests further confirm the serological homogeneity of the type A strains (table 3). Absorption with the F type

TABLE 3

Agglutinin absorption tests with antiserum 730 (type A)

ANTIGENS		UNABSORBED	ABSORBED WITH 1064(D)	ABSORBED WITH 864(F)
Type A	18 strains	400*	400	400
Type D	1,068	200	0	100
	1,061	100	0	100
	1,063	200	0	0
	1,064	400	0	100
	1,067	200	0	100
	956	200	200	200
	1,065	400	0	100
Type F	860	100	0	0
	861	50	0	0
	864	50	0	0
	1,010	400	100	0

0 Indicates no agglutination.
* Numbers indicate highest dilution of serum causing agglutination.

TABLE 4

Absorption tests with antiserum 158 (type B)

TYPE	CULTURE NUMBER	158 B NOT ABSORBED	158 B ABSORBED WITH 1064(D)
B	164	400	200
	158	400	400
D	1,061	400	400
	1,064	400	100
	1,067	400	400
	1,068	400	200
F	1,010	100	100
	1,011	100	100
	1,012	100	100

strain failed to remove the agglutinins for the A type but effectively removed the agglutinins for all the F types and partially removed those for the D type as well. Similarly absorption with the D type strain removed none of the A agglutinins but removed the agglutinins for most of the D and F types. Absorption tests with the anti-B serum using a D strain resulted in lowered titer for a B strain and a D strain, with no effect on the homologous B strain and the F strains (table 4). Likewise absorption of the anti-F serum with a D antigen had little effect on the titer for either A, D, or F strains (table 5).

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For the isolation of the different bacteriophages, "mud" from the L. S. U. sugar house was diluted in about an equal portion of water and allowed to settle for 2 days. The supernatant fluid was first filtered through paper and then through a Pasteur-Chamberland filter. The filtrate obtained was inoculated into a series of tubes seeded with the different strains of *L. mesenteroides*. Each tube containing tryptone glucose yeast extract broth was seeded with 1 loopful of one of the strains and inoculated with 1 ml of the filtrate. A control tube without the filtrate was inoculated with each organism. The tubes were observed after

TABLE 5

Agglutinin absorption tests with antiserum 860 (type F)

ANTIGENS		ANTISERUM 860F	
		Not absorbed	Absorbed with 956(D)
Type A	153	50	50
	732	100	50
	1,021	50	50
	1,022	50	50
	1,024	50	50
Type D	1,067	100	0
	835	200	200
	910	200	200
	956	400	0
	320	200	200
Type F	1,010	400	400
	1,011	200	200
	1,012	100	200
	860	400	400
	861	100	200
	862	400	400
	864	200	200
	866	200	200
	867	200	200
	868	50	50
	708	100	100
	711	100	200
	714	50	200

1 and 2 days for lysis, and those which showed no growth of the organism were reinoculated with a growing, young culture. If no growth occurred, the bacteriophage suspension was filtered through a Pasteur-Chamberland filter and, after several repetitions of this procedure, the filtrate was used for the tests.

Considerable difficulty was experienced in isolating the bacteriophages, and only 5 were obtained. Each bacteriophage was tested for the formation of plaques with the homologous organism and then tested against all the strains available. The bacteriophages obtained were for the strains 730 (A type), 700 and 706 (B type), and 200 and 209 (D type). No bacteriophage was obtained for any organism of the F type.

The lytic activity of the bacteriophages was determined by the spot plate

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method. Petri plates were poured with glucose tryptone yeast extract agar (1 per cent agar) and after solidification were allowed to dry for 4 hours in the incubator at 37 C. Then 2 drops of a young broth culture were spread on the surface by means of a glass rod, and the plates were again allowed to dry for 4 hours. Inoculation with phage was effected by depositing a loopful of active filtrate on a certain spot on the plate. All the bacteriophages were tested on the same plate.

The results of this experiment are presented in table 6. It is apparent that each phage tested exhibited type specificity to a considerable degree. Of 105 strains only two were lysed by phages from outside the type. A high degree of strain specificity was noted, however, particularly in the D type.

TABLE 6

Susceptibility of Leuconostoc mesenteroides to bacteriophages

TYPE	STRAINS TESTED	PER CENT POSITIVE REACTIONS WITH PHAGE				
		730(A)	700(B)	706(B)	200(D)	209(D)
A	18	50	0	0	5.5	0
B	31	3.2	41.9	41.9	0	0
D	42	0	0	0	11.9	9.5
F	14	0	0	0	0	0

SUMMARY

Serological and bacteriophagic studies on strains of *Leuconostoc mesenteroides* isolated from cane juice indicated that Faville's type A constitutes a reasonably distinct and homogeneous group, whereas the B, D, and F types are quite heterogeneous. Of the three tests employed the agglutination test was the most useful in showing type relationships.

REFERENCES

Bergey, D. H., Breed, R. S., Murray, E. G. D., and Hitchens, A. P. 1939 Bergey's manual of determinative bacteriology. Williams & Wilkins Co., Baltimore, Md.

Faville, L. W. 1947 A study of gum-forming bacteria isolated from cane juice. Thesis, Louisiana State University.

Hucker, G. J. 1932 Studies on the *Coccacceae*. XVII. Agglutination as a means of differentiating the species of *Streptococcus* and *Leuconostoc*. N. Y. (Geneva) Agr. Expt. Sta., Tech. Bull. 190.

Lancefield, Rebecca C. 1933 A serological differentiation of human and other groups of hemolytic streptococci. J. Exptl. Md., **57**, 571-595.

McCleskey, C. S., Faville, L. W., and Barnett, R. O. 1947 Characteristics of *Leuconostoc mesenteroides* from cane juice. J. Bact., **54**, 697-708.

Neill, J. M., Hehre, E.J., and Jaffe, E. 1941 Serological studies on sugar. II. Reactions of the antiserums of type II pneumococcus and of *Leuconostoc mesenteroides* with cane and beet sugars and with cane juice. Am. J. Hyg., **34**, 65-78.

Neill, J. M., Sugg, J. Y., Hehre, E. J., and Jaffe, E. 1941 Serological studies on sugar. III. Reactions of the antiserums of type II pneumococcus and of *Leuconostoc mesenteroides* with sugar and sirups of palm, maple and sorghum origins and with sauerkraut juice. Am. J. Hyg., **34**, 79-81.

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