JOURNAL OF BACTERIOLOGY
Vol. 88, No. 1, p. 55-59 July, 1964

CULTIVATION OF LEPTOSPIRAE

II. GROWTH AND LYSIS IN SYNTHETIC MEDIUM

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Received for publication 21 February 1964

ABSTRACT

STALHEIM, O. H. V. (University of Wisconsin, Madison), AND J. B. WILSON. Cultivation of leptospirae. II. Growth and lysis in synthetic medium. J. Bacteriol. 88:55-59. 1964.—Differences were found in the ability of leptospirae to grow in a synthetic medium; 43 strains, consisting of 16 serotypes, were tested and designated as either type I or type II. Type I leptospirae did not grow; type II grew and could be subcultured. The lytic effect of several lipids was measured with *Leptospira pomona* and *L. canicola* as representatives of type I and II leptospirae, respectively. *L. pomona* organisms were rapidly lysed by the monoolein of the synthetic medium and by other lipids as well; *L. canicola* cells were consistently more resistant. Although both organisms incorporated similar amounts of label when incubated in the presence of oleic-*1*-C^{14} acid, only *L. canicola* grew in a modified, nonlytic synthetic medium. No differences were found in susceptibility to lysis between virulent and avirulent *L. canicola* organisms. Mutant type I leptospirae grown in synthetic medium had increased resistance to lysis by surface-active agents; they were poorly agglutinated by antiserum. The role of protein in the growth and antigenicity of type I leptospirae is discussed.

Yanagawa and Wilson (1962) classified 60 strains of leptospirae by their ability to grow in a medium which had been boiled after the addition of 10% rabbit serum. Type I leptospirae did not grow and type II grew, but the growth-limiting agent was not identified. Yanagawa and Wilson (1962) selected mutants of type I which grew in the boiled serum medium. The present report demonstrates that we cannot cultivate type I organisms in synthetic media without resorting to the use of mutant strains. Therefore, we have searched for physiological differences between type I and type II leptospirae by use of *Leptospira pomona* (type I) and *L. canicola* (type II). We have compared their susceptibility to lysis by surface-active agents and measured their utilization of oleic acid.

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MATERIALS AND METHODS

Cultures of leptospirae were maintained in the phosphate buffer-rabbit serum medium of Johnson and Wilson (1960). A 1-ml amount of a 7- to 10-day-old culture (about 10^8 organisms per ml) was inoculated into duplicate tubes containing 10 ml of boiled serum medium (Yanagawa and Wilson, 1962), a synthetic medium (198E) devised by Vogel and Hutner (1961), and a synthetic medium containing Tween 60 and Tween 80 (TSM; Stalheim and Wilson, 1964). The components of the synthetic media are listed in Table 1. The cultures were incubated at 30 C. Those cultures that grew were subcultured at least four times before the results were recorded. The amount of leptospiral growth was evaluated by direct microscopic counts on samples from duplicate tubes by use of a Petroff-Hausser bacterial counting chamber.

Cultures of mutant leptospirae were selected in boiled serum medium as described by Yanagawa and Wilson (1962), and were maintained in TSM by transfers at intervals of 7 to 10 days. Their agglutinability was determined by the microscopic agglutination (MA) test by use of serial, tenfold dilutions of known, homologous rabbit antiserum. Leptospirae were heat-killed by immersing cultures in a water bath at 56 C for 30 min.

Leptospiral lysis was measured by direct microscopic counts of organisms suspended in the two synthetic media and in boiled serum medium. Leptospirae were sedimented from 4 ml of 7- to 10-day-old cultures in phosphate

TABLE 1. *Composition of synthetic media**

Component	Vogel and Hutner (198E)	Stalheim and Wilson (TSM)
KH_2PO_4	10.0	10.0
$MgSO_4$	40.0	40.0
Ca^{++}	0.04	0.04
Ferrous ammonium sulfate	0.65	—
Ferric ammonium citrate	—	0.55
$MnSO_4 \cdot H_2O$	0.045	0.045
$ZnSO_4 \cdot 7H_2O$	0.66	0.66
$CuSO_4 \cdot 5H_2O$	0.04	0.04
$CoCl_2$	0.02	0.04
Sodium acetate $\cdot 3H_2O$	5.0	—
$(NH_4)_2SO_4$	20.0	25.0
Hydroxyethylenediamine-tetraacetic acid	2.0	2.0
Monoolein	2.0	—
Tween 60	—	2.0
Tween 80	—	2.0
Thiamine HCl	0.04	0.04
Vitamin B_{12}	0.00002	0.00002
Biotin	—	0.02
Nicotinic acid	0.02	0.002
DL-Calcium pantothenate	0.02	0.002
Pyridoxine HCl	0.02	0.002
Aspartic acid	10.0	7.5
Asparagine	—	3.0
Glutamic acid	10.0	3.0
Glycine	1.0	0.3
Histidine	25.0	3.0
Isoleucine	1.0	0.3
Leucine	1.0	0.3
Methionine	0.5	0.3
Phenylalanine	5.0	0.6
Proline	2.0	0.6
Alanine	10.0	—
Serine	1.0	—
Tryptophan	2.0	—
Valine	5.0	—

* Medium 198E contained mostly DL-amino acids; TSM contained only L-amino acids. Concentrations are given in mg/100 ml.

buffer-rabbit serum medium by centrifugation (10,000 × *g* for 30 min), and resuspended in 10 ml of test medium. Bacterial counts were performed on samples of the mixtures immediately after suspension, and after incubation at 30 C for 2, 24, and 48 hr. Several fatty acids, methyl oleate, sodium oleate, triolein, Tween 60, and Tween 80 were singly added to medium 198E in place of monoolein, and the lytic effect on *L. pomona* and *L. canicola* was determined. Different concentrations of oleic acid (grade A, Calbiochem) were substituted for the monoolein of synthetic medium, and the minimal lytic concentrations for *L. pomona* and *L. canicola* were determined. Lysis was considered to have occurred if no organisms were observed in 80 small squares of the counting chamber after 48 hr of incubation.

Utilization of oleic acid by *L. pomona* and *L. canicola* was measured in the presence of oleic-*1-C*^{14} acid, when substituted for the monoolein of medium 198E in nonlytic amounts. At intervals of 10, 70, 130, and 1,200 min, samples were removed, and the radioactivity of the sedimented cells and the supernatant medium was determined as previously described (Stalheim and Wilson, 1964). The volatile material was taken as the difference between the radioactivity of the initial suspending medium and of the cells and supernatant medium after incubation.

RESULTS

The following leptospiral serotypes, with the number of strains of each serotype in parentheses, were cultivated in medium 198E, TSM, and boiled-serum medium (type II leptospirae): *L. canicola* (8), *L. hebdomadis* (1), *L. australis* (2), *L. bataviae* (1), *L. biflexa* (1), *L. semaranga* (1), and *L. andaman* (1). The following did not grow (type I leptospirae): *L. pomona* (11), *L. icterohaemorrhagiae* (4), *L. ballum* (3), *L. autumnalis* (4), *L. grippotyphosa* (2), *L. pyrogenes* (1), *L. sejroe* (1), *L. hardjo* (1), and *L. hyos* (1). After the first subculture, positive cultures contained 10^7 to 3.0×10^7 organisms per ml in medium 198E, 3.6×10^7 to 4.0×10^7 in TSM, and 10^8 to 3.0×10^8 in boiled-serum medium.

The failure of type I leptospirae to grow in medium 198E and boiled-serum medium was correlated with the disappearance (lysis) of organisms suspended in these media. Numbers of organisms per ml in medium 198E decreased during 2 hr of incubation to less than one-half the numbers immediately after suspension, and fewer than 2×10^6 per ml remained after 48 hr. Boiled-serum medium lysed leptospirae less rapidly than did medium 198E, but fewer than 2×10^6 organisms per ml remained after 48 hr of incubation. Leptospiral growth did not occur when 1-ml volumes of lysed preparations were added to tubes containing 10 ml of phosphate

buffer-rabbit serum medium. The lytic agent of medium 198E was monoolein, for that medium made without monoolein was not lytic. TSM and medium 198E, supplemented with 10% rabbit serum, were nonlytic.

The lytic effect of different fatty acids, monoolein, triolein, sodium oleate, Tween 60, and Tween 80, when substituted for the monoolein of medium 198E, on *L. pomona* and *L. canicola* is shown in Table 2. *L. pomona* organisms were rapidly lysed when suspended in medium containing C_8, C_{10}, C_{12}, C_{14}, or C_{20} fatty acids, unsaturated C_{18} fatty acids, ricinoleic acid, or monoolein. C_3, C_6, C_{11}, and C_{17} fatty acids and sodium oleate were moderately lytic; saturated C_{16} and C_{18} fatty acids were slightly lytic, whereas triolein, Tween 60, and Tween 80 were only very slightly lytic. *L. canicola* organisms were more resistant to lysis. C_3, C_6, C_8, C_{10}, C_{11}, unsaturated C_{18}, and C_{20} fatty acids, and ricinoleic acid were moderately lytic; C_{12}, C_{14}, C_{16}, C_{17}, and saturated C_{18} fatty acids, triolein, and sodium oleate were slightly lytic, whereas monoolein, Tween 60, and Tween 80 were nonlytic. The minimal lytic concentration of oleic acid for *L. pomona* organisms was 0.01 mg/ml, and for *L. canicola* organisms was 0.08 mg/ml.

No differences were found in susceptibility to lysis between virulent and avirulent leptospirae. *I. canicola* strain Dog-L organisms, which were nonlethal for hamsters in doses of 10^8, and *L. canicola* strain Moulton organisms, which had an LD_{50} value for hamsters of less than 50 organisms, were lysed by the same concentration of oleic acid.

L. pomona could not be cultivated in the nonlytic, modified synthetic medium containing Tweens 60 and 80 (TSM). After the first transfer, moderate growth was supported by serum contained in the inoculum, but macroscopic evidence of growth did not appear after a second transfer. When these cultures were examined microscopically after 14 days of incubation, relatively small and sluggish organisms were observed.

Mutant cultures of *L. pomona*, *L. icterohaemorrhagiae*, *L. autumnalis*, and *L. grippotyphosa*, selected in boiled-serum medium, were capable of growth in medium 198E and TSM; the amount of growth equalled that of type II leptospirae. They retained the capability after four transfers in native serum medium. The minimal, lytic concentration of oleic acid for mutant leptospirae was 0.08 mg/ml. When mutant cultures were mixed with homologous antiserum in the MA test, incomplete agglutination occurred only in serum dilutions of 1:10 and 1:100. At the latter dilution, fewer than one-half of the cells were agglutinated into loose strands and threads. Agglutinability was not improved by washing the cells three times in phosphate buffer. After a few transfers in phosphate buffer-rabbit serum, complete agglutinability was restored.

TABLE 2. *Per cent lysis of Leptospira canicola and L. pomona by fatty acids, monoolein, Tween 80, Tween 60, sodium oleate, and triolein**

Medium† plus	*L. canicola*			*L. pomona*		
	2‡	24	48	2	24	48
Control	0	0	5	0	0	5
Butyric acid	0	5	33	10	20	60
Caproic acid	0	30	60	12	40	82
Caprylic acid	10	45	58	48	72	100
Capric acid	8	22	48	82	100	100
Undecanoic acid	10	20	52	60	65	85
Lauric acid	4	10	10	20	65	100
Myristic acid	6	18	18	55	75	100
Palmitic acid	4	10	12	18	20	24
Heptadecanoic acid	0	4	10	34	70	82
Oleic acid	0	5	15	84	98	100
Monoolein	0	5	0	85	96	100
Triolein	2	4	15	4	4	15
Methyl oleate	0	0	0	0	0	24
Tween 80	0	0	0	0	6	10
Tween 60	0	0	0	0	0	5
Sodium oleate	0	16	32	10	14	52
Ricinoleic acid	8	22	52	64	80	100
Linoleic acid	19	20	42	80	100	100
Linolenic acid	8	20	55	85	96	100
Stearic acid	0	4	16	10	18	24
Arachidic acid	10	50	65	20	30	100
Arachidonic acid	8	45	75	16	32	100

* Leptospirae were sedimented by centrifugation from 4 ml of cultures in phosphate buffer-rabbit serum medium, resuspended in 10 ml of medium, and microscopically counted. Further counts were made after incubation at 30 C for 2, 24, and 48 hr, and the percentage of lysis was recorded as compared with the initial count.

† Medium 198E without monoolein plus 2 mg per 100 ml of each additive.

‡ Time of incubation (hr).

TABLE 3. *Per cent utilization of oleic-1-C^{14} acid by Leptospira canicola and L. pomona*

Organism*	After incubation for			
	10 min	70 min	130 min	1,200 min
L. canicola cells	15.6	27.0	30.5	34.5
Supernatant medium	74.2	43.1	37.8	22.7
Volatile material	10.2	29.9	31.7	42.8
L. pomona cells	12.9	30.5	34.3	36.0
Supernatant medium	69.8	44.0	42.3	28.5
Volatile material	17.3	25.5	23.3	35.5

* Leptospirae were sedimented from 15 ml of *L. canicola* and *L. pomona* cultures in rabbit serum medium, resuspended in 15 ml of synthetic medium containing 0.2 μc/ml of oleic-*1*-C^{14} acid substituted for monoolein, and incubated at 30 C. At intervals, 2-ml samples from each mixture were diluted in 0.25% extracted rabbit albumin and, after centrifugation, the radioactivity of the supernatant medium and the sedimented organisms was determined and calculated in percentage of activity of the original medium (6,600 counts per min per ml). The volatile material was taken as the difference between the radioactivity of the original medium and that of the supernatant medium and cells.

When sedimented *L. pomona* and *L. canicola* organisms were resuspended in medium containing radioactive oleic acid (Table 3), progressive incorporation of C^{14} label occurred over an incubation period of 1,200 min, when the distribution of the label was as follows: *L. pomona* cells, 28%; supernatant medium of *L. pomona* cultures, 35%; and volatile material, 37%; *L. canicola* cells, 34.5%; supernatant medium of *L. canicola* cultures, 22.7%; and volatile material, 42.8%. Heat-killed *L. pomona* and *L. canicola* organisms did not passively bind oleic acid when incubated in medium containing oleic-1-C^{14} acid for 60 min.

DISCUSSION

Certain strains of leptospirae, which have been classified as type I because they do not grow in a boiled rabbit serum medium (Yanagawa and Wilson, 1962), also do not grow in a synthetic medium containing monoolein as a fatty acid source, or in a modified synthetic medium containing Tweens substituted for monoolein. Type II leptospirae grow in all three media, but much better growth occurs in the boiled rabbit serum medium. During investigations on the nature of the growth-limiting component of a synthetic medium, it was found that type I leptospirae are rapidly lysed by monoolein and by other lipids as well. The lytic activity of fatty acids is related to the number of carbon atoms and double bonds. The growth-limiting agent in boiled serum medium is presumably a lytic concentration of free fatty acids released from serum proteins by the boiling process. Heating serum albumin to 100 C destroys its oleic acid-binding capacity (Davis and Dubos, 1947).

Synthetic medium, containing the minimal concentration of monoolein required to support growth of type II leptospirae, lyses type I organisms, and their protein requirement for growth would appear to be related to the detoxifying effect of serum albumin (Davis and Dubos, 1947). This belief is supported by the report of Johnson and Gary (1963), who studied the fatty acid nutritional requirements of a type I leptospire, *L. pomona*. Maximal growth was obtained with palmitic, heptadecanoic, stearic, or oleic acids if the fatty acids were detoxified by the addition of 0.8% extracted rabbit albumin; no growth occurred without albumin. However, we cannot cultivate type I leptospirae in a nonlytic synthetic medium, TSM, which supports the growth of type II leptospirae. Smith and Boughton (1960) studied the protein requirement for growth of pleuropneumonia-like organisms and concluded that the role of protein involves neutralization of the lytic activity of necessary surface-active factors such as oleic acid, as well as regulation of the uptake of an essential nutrient, cholesterol. The protein requirement of type I leptospirae may involve "feeding" the fatty acid molecule, a part of it, or some other growth factor. Type I and II leptospirae may differ in their requirements for protein-bound vitamin B_{12} (Babudieri, 1961; Ellinghausen and McCullough, 1962).

Kodicek (1949) hypothesized that the effects of unsaturated fatty acids on bacteria depend upon the nature of the cell wall to which they are adsorbed. Natural constituents of the cell act as detoxifiers, but above a certain concentration the detoxifying substances are neutralized, and it becomes necessary to add detoxifiers, such as albumin, to the medium. The lipase activity of our test organisms has not been determined, and we do not know whether lysis is due to the

fatty acid *per se*, or to products formed near the cell surface by leptospiral lipase (Kemenes and Lovrekovich, 1959). The greater susceptibility of type I leptospirae to lysis by fatty acids, as compared with type II organisms, may be due to less capacity to neutralize adsorbed fatty acids, or to greater lipase activity.

Mutant type I leptospirae grown in boiled serum medium exhibit no changes in serological behavior (Yanagawa and Wilson, 1962), but after cultivation in TSM, we find that they are poorly agglutinated by known antiserum. Group A streptococci synthesize M protein in a medium containing reduced crystalline ovalbumin (Slade and Slamp, 1955). Recently, certain of the group A streptococci were successfully cultivated in synthetic medium (Mickelson, 1963). These organisms, when grown in synthetic medium, lack the M protein which is essential in serological typing (Slade and Mickelson, *personal communication*). Fox (1961) reported that small peptides from trypsinized casein stimulate the synthesis of type A streptococcal proteins. The decreased agglutinability of leptospirae when grown in synthetic medium suggests a protein requirement for the synthesis of leptospiral agglutinogens.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant E-1664 from the National Institutes of Health; the senior author was a postdoctoral fellow of the National Institutes of Health.

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