INTERNATIONAL JOURNAL OF LEPROSY Volume 49, Number 2
Printed in the U.S.A.

Parameters Influencing the *in vitro* Growth of *Mycobacterium lepraemurium*[1]

Françoise Portaels and Stefaan R. Pattyn[2]

Mycobacterium lepraemurium (*M. lm*) can now readily be grown *in vitro* on 1% Ogawa medium ([3,5,8,10,11,12,13]). Many attempts have been made to define the factors influencing this *in vitro* growth with the hope of improving the composition of the medium or conditions of incubation because it is still not possible to obtain growth from small inocula ([9,12]). This paper summarizes our more recent efforts along these lines.

MATERIALS AND METHODS

M. lm strains were those previously used ([12]), UL, CL, and HS (the latter corresponds to the Douglas strain which was mislabeled in our previous publication as the Hawaiian strain) together with the following Japanese strains obtained from T. Mori: Keishicho, Osaka, Odessa, and Hawaiian. Media were inoculated either by transfering bacteria with a 2 mm platinum loop from an *in vitro* grown culture or with suspensions containing at least 10^9 bacilli per ml prepared from livers of mice injected intravenously with a strain of *M. lm*.

To study the influence of the pH of the medium on the growth of *M. lm*, Ogawa egg yolk media were prepared with KH_2PO_4 at 8, 7, 6, 5, 4, 3, 2, and 1% producing media with pH values varying from 5.5 to 6; pH gradients were also obtained with Ogawa egg yolk media in which KH_2PO_4 was replaced by either Sorensen's phosphate buffer (pH range of final media from 6 to 6.7) or by McIlvaine's citrate-phosphate buffer (pH range of final media from 4.6 to 6.7). The same media were also prepared with whole eggs giving a final pH varying from 5.9 to 7 with the different concentrations of KH_2PO_4, a pH range from 7 to 7.8 with Sörensen's phosphate buffer, and from 4.8 to 7.2 with McIlvaine's citrate-phosphate buffer. All pH values given were measured before coagulation.

Glycerol as a carbon source was tested at final concentrations of 10, 5, 2, 1, 0.5, 0.1, 0.05, and 0% in 1% Ogawa egg yolk medium (OEY). Acetate was tested at 1% final concentration, either added to OEY or replacing the glycerol.

Sodium salts of fatty acids, as indicated in Table 5, were added at 25 and 125 nM to OEY. They were prepared in a manner analogous to Dubos oleic acid albumin supplement: the salts were added to 0.05 N NaOH, agitated for 30 min, and added to 5% solution of bovine albumin (fraction V) containing 5% glycerol. Tween 80 was tested at a final concentration of 2% in OEY. The pH of these media was always 6.0–6.1 before coagulation.

Cultures were incubated in parallel at 33° and 37°C in standard incubators and incubators with 90% humidified air with and without the addition of 5% CO_2. Tubes had rubber stoppers transpierced with 19 gauge disposable needles.

The growth on the different media was judged by comparison with growth obtained on standard 1% Ogawa egg yolk medium, expressing the results as the number of tubes with growth/number of tubes inoculated. Tubes were observed for periods of 3 to 6 months.

RESULTS

Since the different strains of *M. lm* were identical and a number of cultures were lost after contamination by molds, we only present the combined results obtained on all the strains.

Effect of temperature, aeration, CO_2, and humidity. Growth was more rapid at 37°C

[1] Received for publication on 28 November 1980; accepted for publication in revised form on 24 February 1981.

[2] F. Portaels, Dr. Sc., Chef de Travaux, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerpen, Belgium; S. R. Pattyn, M.D., Professor of Medical Microbiology, University of Antwerp, Professor of Tropical Microbiology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerpen, Belgium. Reprint requests to Dr. Portaels.

TABLE 1. *Growth of* M. lepraemurium *at different pH on: A, egg yolk medium and B, whole egg medium. Results expressed as number of culture tubes with growth/number of tubes inoculated (% positive).*

	5.0–5.1	5.2–5.3	5.4–5.5	5.6–5.7	5.8–5.9	6.0–6.1	6.2–6.3	6.4–6.5	6.6–6.7	6.9–7.0
A	0/5	0/4	2/13	14/20	13/13	27/27	8/10	0/2	0/5	—
	(0)	(0)	(15)	(70)	(100)	(100)	(80)	(0)	(0)	—
B	—	0/5	0/6	5/10	5/11	5/16	0/11	0/8	0/2	0/11
	—	(0)	(0)	(50)	(45)	(31)	(0)	(0)	(0)	(0)

than at 33°C; increased CO_2 was without effect; aeration in a humidified atmosphere was necessary. This was best obtained by the use of rubber stoppers transpierced with 19 gauge injection needles; loose screw caps gave irregular results because of the impossibility of standardizing aeration. Growth was greatly impaired or absent in tubes incubated at 37°C with tight rubber stoppers or with transpierced stoppers at 37°C in an unhumidified atmosphere.

Effect of pH and egg white. The effect of pH was independent of the buffer used since there was no difference in growth for identical pH values. Table 1 therefore presents the combined results of all *M. lm* stains on media with all three buffers.

It can be seen that on egg yolk medium the optimal pH is from 5.8 to 6.3 with 100% positivity between 5.8 and 6.1. Growth is already much impaired at pH 5.6–5.7, the difference between 5.6–5.7 and 5.8–5.9 being statistically significant. There is still some growth at pH 5.5 but none below 5.4 or above 6.3. The inhibitory effect of egg white is striking. Even at pH values that are optimal for egg yolk, there is growth in fewer than 50% of the tubes containing whole eggs. This difference is highly significant.

Effect of glycerol, acetate, and Tween 80. Table 2 shows that either absence or high concentrations (5%, 10%) of glycerol in the medium are growth inhibitors. The optimal concentration is between 0.5 and 2%, the difference observed for growth between 0.1 and 0.5% and between 2 and 5% being highly significant.

Table 3 shows that 1% acetate in the presence or absence of glycerol significantly inhibits the *in vitro* growth of *M. lm*. The addition of 2% Tween 80 (Table 4) has no appreciable effect.

Effect of fatty acids. The effects of sodium salts of the fatty acids tested are given in Table 5. Not only was there no stimulatory effect, but in most cases these substances were even inhibitory. All the differences observed are highly significant except between controls and 25 nM caprylate.

DISCUSSION

In the present work four Japanese strains of *M. lm* were studied in parallel with those previously handled ([12]). No differences between these strains were observed. Optimal

TABLE 2. *Effect of different concentrations of glycerol on the* in vitro *growth of* M. lepraemurium.

	Final concentrations of glycerol (%)							
	0	0.05	0.1	0.5	1	2	5	10
Positive cultures	3	20	21	38	10	24	1	0
Total	41	31	31	39	11	24	19	15
% positive	7	65	68	97	91	100	5	0

TABLE 3. *Effect of 1% Na acetate on the* in vitro *growth of* M. lepraemurium *on 1% Ogawa egg yolk medium (OEY) (A) and on 1% OEY without glycerol (B).*

	1% OEY	Acetate	
		A	B
Positive cultures	30	16	4
Total	38	41	18
% positive	79	39	22

TABLE 4. *Effect of 2% Tween 80 on the* in vitro *growth of* M. lepraemurium.

	1% OEY	2% Tween
Positive cultures	38	31
Total	66	64
% positive	58	48

conditions for *in vitro* growth as defined by the present study are egg yolk medium with 0.5 to 2% glycerol, at a pH between 5.8 and 6.2, and incubation of aerated tubes at 37°C in 90% humidified air.

The most important and unexpected finding of the present study is the narrow (0.5 unit) acid pH range allowing *in vitro* growth of *M. lm*. Ogawa and Hiraki ([7]), studying different components of the Ogawa medium, also made important observations on the influence of pH. They observed growth over a wider range: between 5.6 up to exceptionally 7, with an optimum between 5.6 and 6.4. It should be noted that the pH values optimal for *in vitro* multiplication of *M. lm* correspond closely with those at which Hart and Valentine ([2]) observed elongation of the organism in liquid media. It may well be that the reason why this elongation occurred without ensuing multiplication was the absence of adequate aeration. Our results on optimal pH for growth are in contradiction with those mentioned by Nakamura, who found pH 6.5 to 6.8 to be optimal ([6]). On the other hand, these authors worked with liquid media.

A parallel may be sought between the acid pH at which *M. lm* grows *in vitro* and the acid pH of the intracellular phagolysosomes in which *M. lm* multiplies *in vivo*. We were unable to find any information on the effect of pH on the multiplication of mycobacteria in general. It seems that the study of this parameter has been neglected, pH 7 being empirically accepted as a universal optimal pH. Studies on the role of the pH on mycobacterial growth are currently underway in our laboratory. Preliminary results show that different species multiply *in vitro* within different pH ranges, some narrow, others wide.

Another important inhibiting factor for the *in vitro* cultivation of *M. lm* is egg white. Its incorporation into the medium significantly inhibits growth even at optimal pH. This may be the result of sequestration of iron or other ions.

Glycerol should be present in small concentrations from 0.5 to 2%. Addition of 2% Tween 80 to the medium had no beneficial effect.

The effect of acetate, Tween 80, and fatty acids was studied in connection with the results obtained by Carmargo, *et al.* ([1]), who found by radiometric studies that acetate was a better carbon source than glycerol, that incubation at 30°C was superior to 37°C, and that Tween 80 and oleic acid stimulated the metabolism of *M. lm*. We were unable to confirm any of these findings. Different fatty acids were tested because McCarthy ([4]) had found that palmitate was an essential growth factor for *M. avium*. Stimulating or inhibiting conditions are not only reflected by the percentage of positive tubes but also by the number and size of colonies which are more numerous and larger in favorable than in less favorable conditions. Ogawa medium, however, is still not optimal since, as described previously ([12]), large inocula are still needed to obtain *in vitro* growth of *M. lm*.

SUMMARY

Investigations were done on the role of several parameters of incubation, carbon sources, fatty acids and Tween 80 on the *in vitro* growth of *M. lm* on Ogawa medi-

TABLE 5. *Effects of sodium salts of fatty acids (25 or 125 nM) on the* in vitro *growth of* M. lepraemurium.

	1% OEY	Oleate		Caproate		Caprylate		Palmitate	
		25	125	25	125	25	125	25	125
Positive cultures	29	17	16	13	13	5	2	5	2
Total	30	27	29	24	25	6	9	8	8
% positive	97	63	56	54	52	83	22	63	25

um. It was found that incubation of aerated tubes at 37°C in a humidified atmosphere was optimal. Glycerol is necessary in the medium at concentrations of 0.5 to 2%. Acetate inhibits growth; there is no beneficial effect from Tween 80 or sodium salts of oleic, caproic, caprylic and palmitic acid. The pH of the medium is very critical since optimal growth occurs only between pH 5.8 and 6.3 on Ogawa egg yolk medium.

RESUMEN

Se investigó el efecto de varias condiciones de incubación, de la fuente de carbono, de diversos ácidos grasos y del Tween 80, sobre el crecimiento *in vitro* del *M. lm* en el medio de Ogawa. Se encontró que las condiciones óptimas de incubación incluyen aereación, 37°C y atmosfera húmeda. El glicerol es necesario en el medio a concentraciones del 0.5 al 2%. El acetato inhibe el crecimiento; no hay efectos benéficos del Tween 80 ni de las sales sódicas de los ácidos oleico, caproico, caprílico y palmítico. El pH del medio es crítico puesto que el crecimiento óptimo en el medio de Ogawa con yema de huevo sólo ocurre entre pH 5.8 y 6.3.

RÉSUMÉ

L'infuence de divers paramètres d'incubation, de sources de carbone, acides gras et du Tween 80 fut étudiée sur la multiplication *in vitro* de *M. lm* sur milieu de Ogawa. Les conditions optimales d'incubation sont des tubes aérés incubés à 37°C dans une atmosphère humide. La glycérine est nécessaire à une concentration de 0.5 à 2%. L'acétate inhibe la croissance tandis que le Tween 80 et les sels sodiques des acides oléique, caproique, caprylique et palmitique sont sans effet. Le pH du milieu par contre est très important: la multiplication *in vitro* est optimale sur milieu de Ogawa à pH variant entre 5.8 et 6.3. De plus le blanc d'oeuf exerce un effet inhibiteur sur la croissance de *M. lm.*

Acknowledgments. This work was supported by the Leprosy Unit, WHO, Geneva, and the Damiaanfonds, Brussels. We wish to thank A. Franken and L. Van den Breen for technical assistance.

REFERENCES

1. Camargo, E. E., Larson, S. M., Tepper, B. S. and Wagner, H. N. Radiometric studies of *Mycobacterium lepraemurium*. Int. J. Lepr. **44** (1976) 294–300.
2. Hart, P. D'A. and Valentine, R. C. Growth (without multiplication) of *Mycobacterium lepraemurium* in cell-free medium. J. Gen. Microbiol. **32** (1963) 43–53.
3. Koseki, Y., Anchi, T. and Okamoto, S. Ogawa's bacillus: slow growing mycobacteria isolated from mice previously infected with murine leprosy bacillus. I. *In vitro* cultivation and animal inoculation. La Lepro **41** (1972) 127–136.
4. McCarthy, C. Effect of palmitic acid on cell division in *Mycobacterium avium*. Infect. Immun. **9** (1974) 363–372.
5. Mori, T. Cultivation of *M. lepraemurium* on the 1% Ogawa's egg yolk medium and animal inoculation with cultivated *M. lepraemurium*. La Lepro **43** (1974) 226–233.
6. Nakamura, M. Quantitative multiplication of *Mycobacterium lepraemurium*. J. Gen. Microbiol. **82** (1974) 385–391.
7. Ogawa, T. and Hiraki, M. Studies on murine leprosy bacillus. V. Growth in relation to concentrations of monopotassium phosphate, sodium glutamate and glycerol in the 1% egg yolk medium and relation to the pH of the medium. Kitasato Arch. Exp. Med. **45** (1972a) 25–31.
8. Ogawa, T. and Hiraki, M. Studies on murine leprosy bacillus. VI. Attempt to cultivate *in vitro* one other strain of *Mycobacterium lepraemurium*: primary isolation of slow growing mycobacteria from mice previously inoculated with the Keishicho strain. La Lepro **41** (1972a) 118–123.
9. Ogawa, T. and Hiraki, M. Studies on murine leprosy bacillus. XII. Reproduction of the disease in mice using small doses of the thirteenth subculture of supposed Hawaiian strain of *Mycobacterium* lepraemurium. La Lepro **43** (1974) 241–249.
10. Ogawa, T. and Motomura, K. Studies on *Mycobacterium lepraemurium*. First Report. Attempt to cultivate *in vitro* the Hawaiian strain of *Mycobacterium lepraemurium*. La Lepro **38** (1969) 246–254.
11. Ogawa, T. and Motomura, K. Studies on murine leprosy bacilli. IV. Attempts to cultivate *in vitro* the Hawaiian strain of *Mycobacterium lepraemurium*. The further report on primary *in vitro* isolation, subcultivation, reproduction test of the disease in mice of slowly growing acid fast organisms, supposedly murine leprosy bacillus. Kitasato Arch. Exp. Med. **44** (1971) 167–183.
12. Pattyn, S. R. and Portaels, F. *In vitro* cultivation and characterization of *Mycobacterium lepraemurium*. Int. J. Lepr. **48** (1980) 7–14.
13. Sato, S. Cultivation trials of *M. leprae* and *M. lepraemurium*, in particular, re-examination of Ogawa's technique. La Lepro **41** (1972) 65–66.