Biochemical Properties of Cultivated
Mycobacterium lepraemurium[1]

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Since it is now possible to cultivate *Mycobacterium lepraemurium* on the 1% Ogawa yolk medium ([7, 8, 15-22, 25]) it is very easy to obtain a lot of cultivated organisms. Biochemical properties of cultivated *M. lepraemurium* were examined to compare with previous results ([9, 11]) relating to the biochemical properties of *in vivo* grown *M. lepraemurium* in our laboratory. In this paper several biochemical properties which are characteristic of *M. lepraemurium* are described, these characteristics are significant conditions for the identification of this mycobacterium.

MATERIALS AND METHODS

Cultivation of M. lepraemurium Hawaiian strain. A stock solution of 800 μg/ml hemin was prepared by the Biberstein and Gills method ([2]) as suggested by Nakamura ([12]) in that 8 mg of hemin were dissolved in 2 ml of trimethylamin and diluted to 10 ml with distilled water. Modified 1% Ogawa yolk medium supplemented with 0.2 ml of hemin stock solution in 100 ml basal medium was used in this study. Malachite green was omitted from the medium to avoid its mixing with the later extraction of the red pigment. Hereafter this medium is designated as the hemin yolk medium. Inoculation of the cultivated *M. lepraemurium* was carried out by transplantation with the colony mass. After about one month, proliferating colonies dispersed with condensed water were spread over the full surface of the medium, and yet one month later full colony growths of *M. lepraemurium* appearing over the whole surface of the medium were collected with a spatular platinum needle.

Preparation of cell free extract. *M. lepraemurium* collected from hemin yolk medium was ground in a little distilled water with a glass rod and then suspended in ten times

the volume of water and washed with centrifugation at 10,000 rpm for 30 minutes. (Distilled water was used to avoid salt solution because of the need for concentration of growth promoting factor from the supernatant in another experiment.) Frozen pieces of the precipitates were placed on a filter paper, and dehydrated overnight in a refrigerator. The dehydrated bacterial mass was ground well in a mortar with quartz sand of the same weight quantity as the bacterial mass. The ground material was suspended in M/10, pH 7.0 Sörensen phosphate buffer ten times the amount of the weights of the wet bacilli and centrifuged for 30 minutes at 12,000 rpm at 4°C. The supernatant was used for the cell free extract.

Measurement of dehydrogenase. Triphenyl-tetrazolium chloride (TTC) was used as an electron acceptor. Dehydrogenase reaction was measured under vacuum condition in Thünberg's tubes, 1 ml of cell free extract or bacterial suspension being placed in the main tube, and 0.2 ml (10^{-5} mol) of TTC together with 0.3 ml (1.5×10^{-6} mol) of substrate in the side arm. After incubation the enzyme reaction was stopped by means of 0.2 ml of 10% trichloroacetic acid, and the formazan was extracted in 5 ml or 10 ml of ethyl acetate. The red color of formazan was determined at the wave length of 480 mμ in a Coleman Junior spectrophotometer.

Measurement of oxygen uptake. Oxygen consumption was measured by the conventional Warburg manometric method.

Measurement of NADH oxidation. A drop of the particulate suspension which was prepared as a precipitate from the cell free extract of *M. lepraemurium* by ultracentrifugation was added to NADH solution and the decrease of optical density of NADH at the wave length of 340 mμ was determined every minute.

Cultivation of acid-fast bacilli. As *M. lepraemurium* grows only on the 1% Ogawa yolk medium, the other acid-fast bacilli were also cultivated on this medium. Malachite green was omitted from the medium so as

not to interfere with the extraction of red pigment produced on the medium. Each acid-fast bacillus was harvested at its maximum growth.

Measurement of cytochromes. The particle fraction which was obtained as the precipitate in ultracentrifugation at 40,000 rpm for 60 minutes from the previously described cell free extract, was suspended in Sörensen phosphate buffer, M/10, pH 7.0. Oxidoreductive difference spectrum of cytochromes of the particle suspensions were measured by a Shimazu multipurpose spectrophotometer reducing with hydrosulfite or NADH in the case of *M. lepraemurium* and reducing with hydrosulfite only in the case of the other acid-fast bacilli.

Discrimination of the pigment produced in the medium. After the acid-fast bacilli were removed from the 1% Ogawa and 1% Ogawa yolk media, ultraviolet light from a Toshiba discriminative apparatus was used to illuminate the media in a dark room. The degree of red fluorescence characteristic in porphyrin was judged by the naked eye.

Extraction of porphyrin from the medium. After the harvest of acid-fast bacilli, water extractable substances were removed from the used media with 20 ml of distilled water overnight in a refrigerator, and then the porphyrin produced on the medium was extracted with 3 ml of 1 N HCl. According to the method of Nicholas *et al* ([13]) five grams of talc powder suspended in 2 N HCl were poured into a small column and aspirated to make a talc column. Porphyrin extracted solution was pooled, filtered with filter paper, poured into the column and aspirated. After the porphyrin was adsorbed on the surface of the talc column, the column was washed with a suitable volume of distilled water to remove the 1 N HCl. Since the talc powder is very efficient in adsorbing the porphyrin, three to five grams of talc powder were enough to adsorb the porphyrin extract from one or two liters. After the talc was pushed out of the column, the red talc having adsorbed porphyrin was cut off and suspended in acetone:water (1:1). Talc powder was removed by centrifugation and filtration with filter paper. The acetone-water porphyrin solution which was obtained by repetition of this procedure was condensed in vacuum by a rotary evaporator.

Paper chromatography of porphyrin. Free porphyrins were developed with a solvent composed of 2,6-lutidine:water (6:4) at 5°C in a cold room for 16 hours according to the ascendence method of Nicholas *et al* ([14]). Porphyrin esters were developed with a solvent composed of chloroform:kerosin (4:2.6) at 25°C for 25 minutes by the method of Chu *et al* ([3]). After the developed paper chromatogram was dried in a dark draft chamber the red fluorescent spots of porphyrins were marked with a pencil on the paper chromatogram under ultraviolet light.

Measurement of absorption spectra of porphyrins. Each porphyrin solution extracted from the medium was applied linealy on the original line of a large Toyo filter paper No. 51, and developed with lutidine-water solvent. Red fluorescent bands and the corresponding positions of blank filter paper were cut off and extracted with 1 N HCl. Absorption spectra of the extract were drawn automatically by a Shimazu multipurpose spectrophotometer using the blank extract as the control.

Determination of protein. Folin's phenol method was used.

Materials. Coproporphyrin III prepared from culture fluid of *Corynebacterium diphteriae* was given by Prof. Yoneda. Hematoporphyrin was a product of Sigma Chemical Corporation. NADH was from Nutritional Chemical Corporation. Protoporphyrin was purchased from Nakarai Chemical Corporation. Hemin and 2,6-lutidine were obtained from Wako Pure Chemical Corporation.

RESULTS

Dehydrogenase activity of cultivated M. lepraemurium. As seen in Table 1, endogenous dehydrogenase activity of whole cells was so strong that reduction of TTC response to substrates was not seen except in sodium laurate. Malate and citrate accelerated the formation of formazan (reduced TTC) in the case of cell free extract of cultivated *M. lepraemurium*. These results were quite the same as that of *in vivo* grown *M. lepraemurium* ([11]).

Respiration of cultivated M. lepraemurium. Cultivated *M. lepraemurium* consumed a considerable amount of oxygen in endogenous respiration, but acceleration of oxygen uptake corresponded to glycerin, and sodium glutamate added substrate did not occur as seen in Figure 1. This phenomenon also was quite similar to observations on *in*

Table 1. *Reduction of triphenyl-tetrazolium-chloride by cultivated* M. lepraemurium.

Substrate	Whole Cell		Cell Free Extract	
1.5×10^{-6} mol	OD	$\triangle$OD	OD	$\triangle$OD
Control	2.80	—	0.16	—
Glycerin	2.80	0	0.15	-0.01
Glutamate	2.60	-0.20	0.11	-0.05
Glucose	2.60	-0.20	0.17	+0.01
Pyruvate	2.65	-0.16	0.15	-0.01
Succinate	2.50	-0.30	0.19	+0.03
α-ketoglutarate	2.35	-0.44	0.17	+0.01
Citrate	2.40	-0.40	0.46	+0.30
Malate	2.50	-0.30	0.48	+0.32
Laurate.	5.20	+2.40	—	—

M. lepraemurium 139 mg/ml   Protein 2816 γ/ml
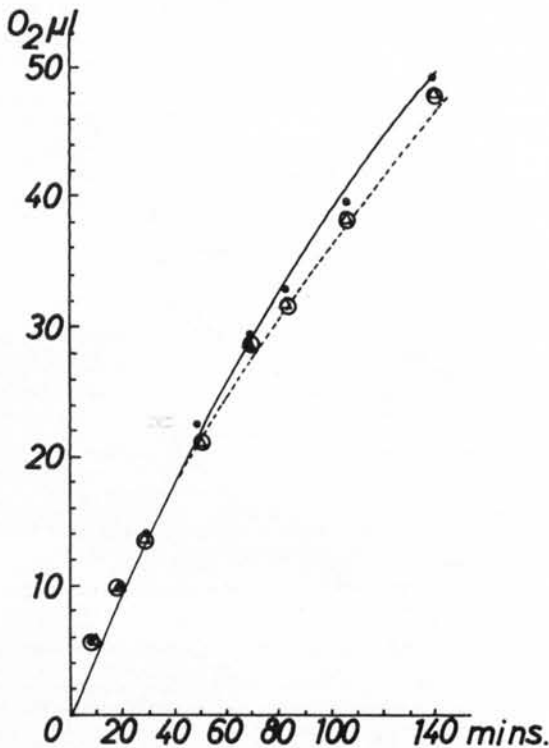Incubation time 2 hr   Incubation time 1 hr

FIG 1. Oxygen consumption of cultivated *M. lepraemurium* by the Warburg manometric method. The amount of 0.2 ml of 20% potassium hydroxide was added in each center well. The amount of 0.2 ml of each substrate 10^{-5} mols was added in the side arm; 2.3 ml of bacterial suspensions (15 mg of dry weights) in M/10 Sörensen phosphate buffer pH 7.0 were added in each main chamber. These vessels were shaken in a water bath at 37.5°C. Control without substrate ●; 10^{-5} mols of glycerin △; 10^{-5} mols of sodium glutamate ○.
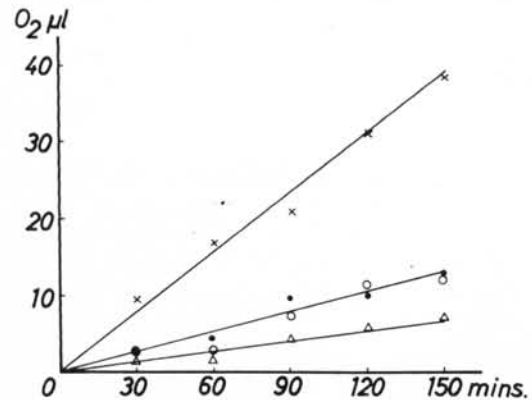

FIG. 2. Oxygen consumption in cell-free extract of cultivated *M. lepraemurium* by Warburg manometric method. Wet weights 3.5 gm of cultivated *M. lepraemurium* were ground with 6 gm of quartz powder and extract with 35 ml of M/10 Sörensen phosphate buffer pH 7.0. Used in this experiment were 2 ml of cell-free extracts (6.64 mg protein). The other conditions were the same as Figure 1. Control without substrate ●; 10^{-5} mols of α-ketoglutarate △; 10^{-5} mols of citrate ○; 10^{-5} mols of NADH X.

vivo grown *M. lepraemurium* [5]. QO_2 of *in vivo* grown *M. lepraemurium* was 4 μl [10] while that of cultivated *M. lepraemurium* was 1.7 μl. The reason for this may be in the mixture of live and dead bacilli found in the culture state. Oxygen consumption was measured in cell free extract by the Warburg manometric method, for the substrates may not penetrate through the cell membrane. As seen in Figure 2, citrate and α-ketoglutarate did not accelerate oxygen consump-
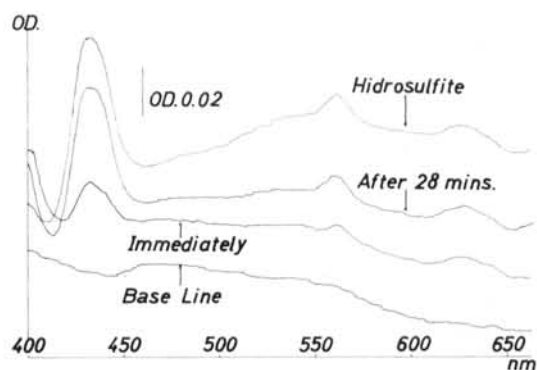
FIG. 3. Difference spectra of cytochromes reduced by hydrosulfite and NADH in particle fraction of cultivated *M. lepraemurium*. The amounts of 3.6 mg/ml protein of particle suspension were used.
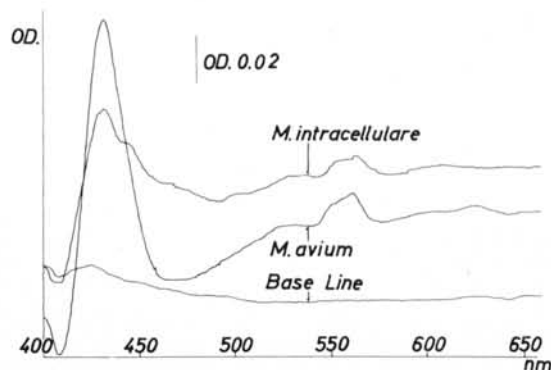

FIG. 4. Difference spectra of cytochromes reduced by hydrosulfite in the particle fraction of *M. avium* Kirchberg and *M. intracellulare* ATCC 15985. The amounts of 3.4 mg/ml and 2.0/ml protein of particle suspension were used respectively in *M. avium* Kirchberg and *M. intracellulare*.

tion, but a little oxygen consumption was seen in NADH. Rapid oxidation of NADH was not seen in the assay system just as it is not seen in *in vivo* grown *M. lepraemurium*, but a slow oxidation which may be dependent on NADH oxidase was measured.

Cytochromes of cultivated M. lepraemurium. As seen in Figure 3, type b_1 cytochrome at the wave length of 561 mμ and type a_2 cytochrome at 625 mμ were observed in the oxidoreductive difference spectra of cultivated *M. lepraemurium*, but type c cytochrome possessing an absorption peak at the neighboring wave length of 550 mμ was not found. These findings were almost the same as previously reported ([10]) except that the absorption spectrum of type a_2 cytochrome in *in vivo* grown *M. lepraemurium* was seen at 630 mμ. Cytochrome c was not detected in even the material reduced with hydrosulfite. Hydrosulfite reduces the hemin which is a contaminant in the particle fraction from the hemin yolk medium while NADH does not reduce the hemin. As the absorption spectrum reduced with NADH resembled that reduced with hydrosulfite, it is clear that the absorption spectrum does not depend on a contamination by hemin added to the medium. Oxidoreductive difference spectrum of cytochromes was measured in some typical acid-fast bacilli to show that no detection of type c cytochrome is a characteristic in cultivated *M. lepraemurium*. As seen in Figures 4, 5, 6 and 7, the typical strains of acid-fast bacilli had an ab-
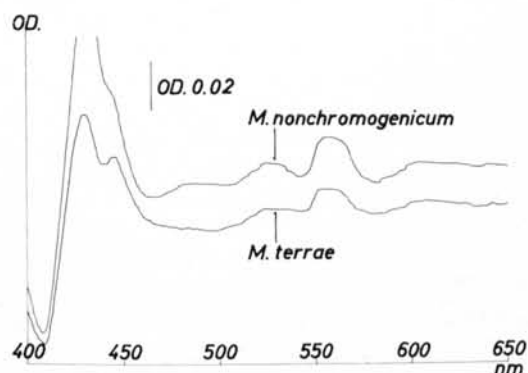

FIG. 5. Difference spectra of cytochromes reduced by hydrosulfite in the particle fraction of *M. terrae* ATCC 15755 and *M. nonchromogenicum* ATCC 19531. The amounts of 1.0 mg/ml and 2.4 mg/ml protein of particle suspension were used respectively in *M. terrae* and *M. nonchromogenicum*.
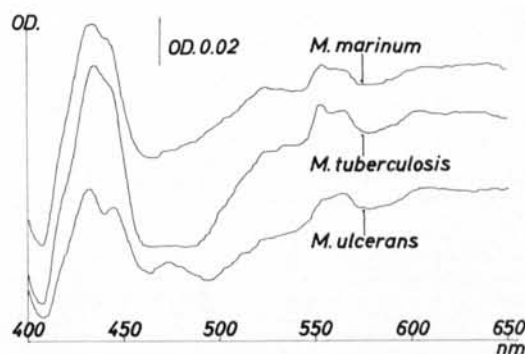

FIG. 6. Difference spectra of cytochromes reduced by hydrosulfite in the particle fraction of *M. ulcerans* NCTC 10417, *M. tuberculosis* H37Rv and *M. marinum* ATCC 927. The amounts of 4.5 mg/ml, 3.7 mg/ml and 2.4 mg/ml protein of particle suspension were used respectively in *M. ulcerans*, *M. tuberculosis* and *M. marinum*.
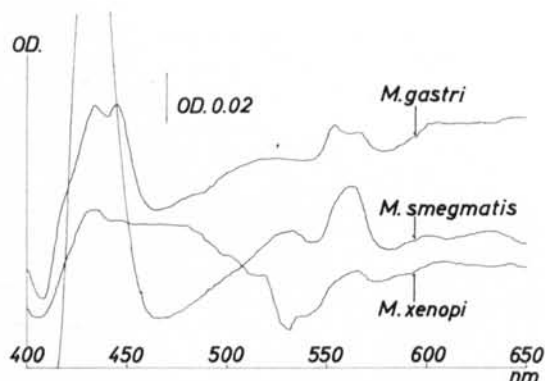
FIG. 7. Difference spectra of cytochromes reduced by hydrosulfite in the particle fraction of *M. xenopi* ATCC 19276, *M. smegmatis* ATCC 14468 and *M. gastri* ATCC 15754. The amounts of 4.2 mg/ml, 5.4 mg/ml protein of particle suspension were used respectively in *M. xenopi*, *M. smegmatis* and *M. gastri*.

sorption peak of type c cytochrome at the wave length of 550 mμ.

Red pigment produced in cultivated M. lepraemurium. When *M. lepraemurium* was cultivated on 1% Ogawa yolk medium, production of brown pigment was observed on the medium under the colony. The pigment was red on 1% Ogawa yolk medium not having malachite green. Red pigment produced by cultivated bacteria is frequently coproporphyrin III. When the red pigment was irradiated with ultraviolet light it emitted a red fluorescence characteristic of porphyrin. On the supposition that the production of the red pigment may be a characteristic of *M.*

Table 2. *Red fluorescence under ultraviolet light on egg media without malachite green.*

Strain	Fluorescence
M. lepraemurium Hawaii	+++
M. avium 4110	+++
M. avium Kirchberg 3717	+++
M. avium ATCC 15769	+++
M. intracellulare ATCC 15985	+++
M. gastri ATCC 15754	+
M. tuberculosis H37Ra	+
M. ulcerans NCTC 10417	±
M. marinum ATCC 927	±
M. bovis Ravenel	-?
M. bovis BCG Takeo	-?
M. kansasii ATCC 12478	-
M. xenopi ATCC 19276	-
M. nonchromogenicum ATCC 19531	-
M. terrae ATCC 15755	-

lepraemurium, production of the red pigment was compared to that of the other acid-fast bacilli. Likewise, the nature of the red pigment was studied for a possible relationship between the red pigment and a growth factor of *M. lepraemurium*. As seen in Table 2, *M. avium* and *M. intracellulare* produced the red pigment just as abundantly on their media as did *M. lepraemurium*. No production of the red pigment was seen with *M. terrae* and *M. nonchromogenicum*, but the other stains produced slight amounts.

Porphyrins extracted from *M. lepraemurium*, *M. avium* and *M. intracellulare* were developed on paper chromatography. The porphyrins of each strain were separated to three spots showing red fluorescence under ultraviolet light. Figure 8 is a schema with
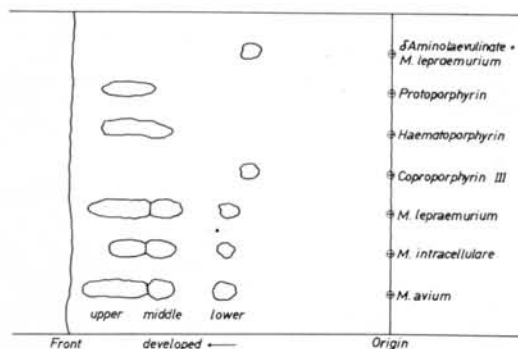

FIG. 8. Paper chromatogram of red pigments from *M. lepraemurium*, *M. intracellulare* and *M. avium*. The paper chromatogram was developed with 2,6-lutidine: water (6:4) at 5°C for 16 hours.

pencil marks around the red fluorescent spots. Large scale paper chromatography was carried out to scan the absorption spectrum. In the region of visible light the absorption spectrum of each porphyrin solution extracted from the three spots with 1 N HCl resembled that of coproporphyrin III, as seen in Figures 9 and 10, i.e., having soret absorption peak at a wave length of 402 mμ and two absorption peaks at 544 mμ and 588 mμ. Porphyrins extracted from *M. avium* and *M. intracellulare* had the same characteristics as seen in Figure 10. While the upper spot was near the Rf value of protoporphyrin which has two carboxyl groups, coproporphyrin having three carboxyl groups did not locate in that Rf. Porphyrin quickly extracted with 1 N HCl was developed with chloroform-kerosin by the method of Chu *et al* ([3]). In this method free porphy-
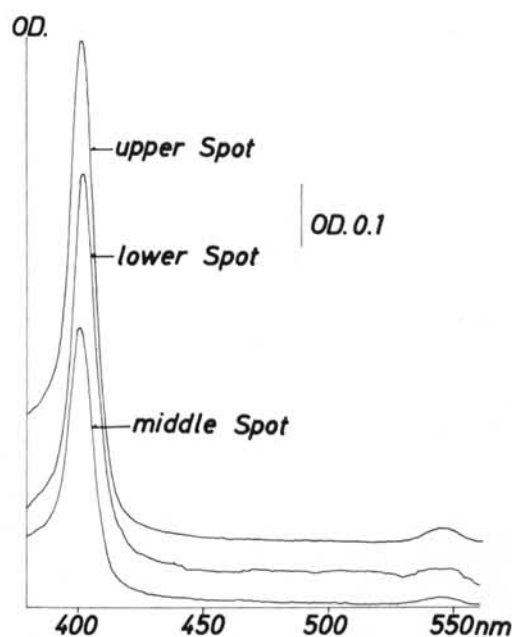
OD.

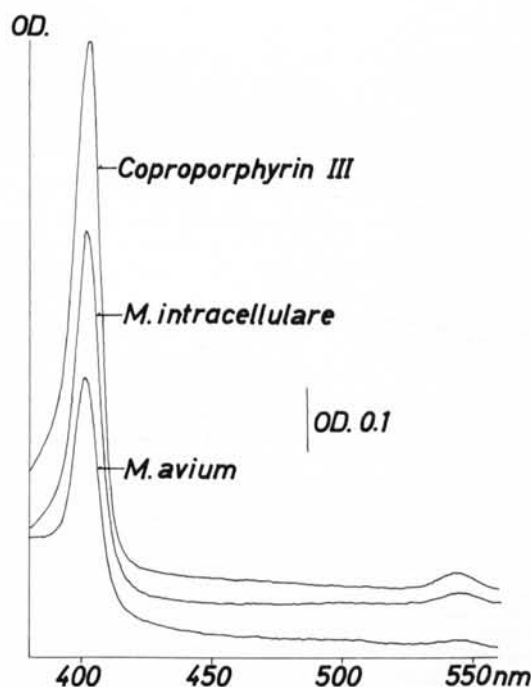FIG. 9. Visible light spectra of red pigments from *M. lepraemurium.*

OD.

FIG. 10. Visible light spectra of red pigment from *M. avium, M. intracellulare* and coproporphyrin III.

rin does not move from the original line, but porphyrin esters move according to their nature. The quickly extracted porphyrin of *M.*

lepraemurium showed the characteristic of porphyrin ester. Free coproporphyrin may be produced gradually by splitting the ester by long extraction with 1 N HCl.

DISCUSSION

Hanks ([6]) and Gray ([5]) reported that *in vivo* grown *M. lepraemurium* did not increase its oxidizing activity with added substrates as determined by the Thūnberg dehydrogenase method and the Warburg manometric method respectively. Segal and Bloch ([23]) reported the same phenomenon in *in vivo* grown *M. tuberculosis*. Since cultivated acid-fast bacilli possess cytochrome c and oxidizing activity in response to added substrates, it was suggested that cultivated *M. lepraemurium* may have cytochrome c and oxidizing activity. However, quite the opposite situation exists in that cultivated *M. lepraemurium* have almost the same characteristics as *in vivo* grown *M. lepraemurium*. Therefore, it must be clarified in the future whether or not *M. tuberculosis* and *M. avium*, which possess cytochrome c in the cultivated state, have cytochrome c in the *in vivo* grown conditions. *M. lepraemurium* cannot be cultivated with a small inoculum on 1% Ogawa yolk medium. The reason for this difficulty in cultivation may relate to lack of cytochrome c.

It was supposed at first that the production of red pigment might be a characteristic of *M. lepraemurium* and that the red pigment might be a growth factor. However, it was found to be an ester of coproporphyrin, i.e., a by-product. *M. lepraemurium* resembles *M. avium* and *M. intracellulare* in porphyrin production on egg medium judging from the listed characteristics for the biochemical classification of mycobacteria ([24]). Alpha-antigen (α -antigen) of *M. lepraemurium* could not be distinguished from that of *M. avium* ([4]). Asami *et al* ([1]) reported that *M. lepraemurium* resembles *M. avium* in the cutaneous reaction produced in sensitized guinea pigs.

SUMMARY

1. Dehydrogenase activity of whole cell of cultivated *M. lepraemurium* is accelerated with sodium laurate, but not with the other substrates.
2. Dehydrogenase activity of cell free extract of cultivated *M. lepraemurium* is accelerated with citrate or malate.

3. There is no acceleration of oxygen consumption corresponding to added substrates in the respiration activity of whole cells of cultivated *M. lepraemurium,* but endogenous QO_2 is 1.7 μl. ·

4. Cell free extract of cultivated *M. lepraemurium* shows slight acceleration of oxygen consumption with NADH, but does not with citrate or α-ketoglutarate.

5. NADH is not oxidized rapidly with the particle fraction of cultivated *M. lepraemurium.*

6. Type b_1 cytochrome having an absorption peak at a wave length of 561 mμ and type a_2 cytochrome having an absorption peak at 625 mμ are detected in an oxidoreductive difference spectrum or particle fraction of cultivated *M. lepraemurium,* but type c cytochrome having the absorption peak at 550 mμ is not seen. Since the other cultivable acid-fast bacilli always have type c cytochrome, nondetection of type c cytochrome is characteristic for *M. lepraemurium.*

7. These cytochromes are reduced with NADH.

8. *M. lepraemurium* produces a red pigment which emits a red fluorescence with ultraviolet light on its 1% Ogawa yolk medium. This phenomenon is a characteristic of *M. lepraemurium, M. avium* and *M. intracellulare.*

RESUMEN

1. La actividad de deshidrogenasa de células enteras de *M. lepraemurium* cultivado, se acelera con laurato de sodio, pero no con otros substratos.

2. La actividad deshidrogenasa de un extracto libre de células de *M. lepraemurium* cultivado, se acelera con citrato o malato.

3. No hay aceleración del consumo de oxígeno correspondiente a los substratos añadidos en la actividad respiratoria de las células enteras del *M. lepraemurium* cultivado, pero el QO_2 endógeno es de 1.7 μl.

4. El extracto libre de células del *M. lepraemurium* cultivado, muestra una ligera aceleración del consumo de oxígeno con NADH, pero no con citrato o alfa-quetoglutarato.

5. La NADH no se oxida rápidamente con la fracción particulada del *M. lepraemurium* cultivado.

6. El citocromo tipo b_1 que tiene un pico de absorción a una longitud de onda de 561 mμ y el tipo a_2 que tiene un pico de absorción a 625 mμ, se detectan en un espectro diferencial oxidoreductivo o fracción particulada del *M. lepraemurium* cultivado, pero el citocromo tipo c, que tiene un pico de absorción a 550 mμ, no se observa. Ya que las otras bacterias ácido-resistentes cultivables siempre tienen citocromo tipo c, la no detección del citocromo tipo c, es característica del *M. lepraemurium.*

7. Estos citocromos se reducen con NADH.

8. El *M. lepraemurium* produce un pigmento rojo que emite una fluorescencia roja con la luz ultravioleta en medio de yema de Ogawa al 1%. Este fenómeno es característico del *M. lepraemurium, M. avium* y *M. intracellulare.*

RÉSUMÉ

1. L'activité en déhydrogénase de la cellule bactérienne entière de *M. lepraemurium* en culture est accélérée par le laurate de sodium, mais pas par d'autres substrats.

2. L'activité en déhydrogénase de l'extrait acellulaire de *M. lepraemurium* en culture est accélérée par le citrate et le malate.

3. Il n'ya pas d'accélération de la consommation en oxygène en rapport avec les substrats ajoutés au milieu, dans l'activité respiratoire de la cellule bactérienne entière de *M. lepraemurium* en culture; le QO_2 endogène s'élève à 1,7 μl.

4. Les extraits acellulaires de *M. lepraemurium* en culture montrent qu'une accélération faible de la consommation en oxygène avec du NADH, mais non pas avec du citrate ou de l'α-cétoglutarate.

5. Le NADH n'est pas oxygéné rapidement avec la fraction particulée de *M. lepraemurium* en culture.

6. Le cytochrome de type b_1, avec pic d'absorption à une longueur d'ondes de 561 mμ, et le cytochrome de type a_2 avec un pic d'absorption à 625 mμ, sont détectés dans le spectre des différences d'oxydoréduction ou dans la fraction particulée de *M. lepraemurium;* le cytochrome de type c, avec pic d'absorption a 550 mμ, n'est pas détecté. Cette absence de détection du cytochrome de type c est caractéristique de *M. lepraemurium,* car les autre bacilles acidorésistants cultivables révèlent toujours du cytochrome de type c.

7. Ces cytochromes sont réduits par le NADH.

8. *M. lepraemurium* produit un pigment rouge qui émet une fluorescence rouge à la lumière ultraviolette en milieu à l'oeuf d'Ogawa à 1 pour cent. Ce phénomène est caractéristique de *M. lepraemurium, M. avium,* et *M. intracellulare.*

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