Fatty Acid Synthesizing Enzyme Activity of Cultured *Mycobacterium lepraemurium*[1]

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For more than half a century, *Mycobacterium lepraemurium* has been well-known as an incultivable mycobacterium as has *Mycobacterium leprae*. Recently it became possible, however, to cultivate *M. lepraemurium* by means of Ogawa's method (11-14). This method presents an invaluable facility for biochemical study of this microorganism, because isolation and collection of its pure and native cells from the cultivation medium are evidently much easier than these procedures from host tissues. Owing to this cultivation, studies on fatty acid biosynthesis of *M. lepraemurium*, which have been almost unknown up to the present, can be carried out and it has been found that this microorganism contains quite a unique pathway for the synthesis of fatty acids as compared with *Mycobacterium smegmatis*, one of the rapid growing mycobacteria. These findings are presented in this paper.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The Hawaiian strain of *M. lepraemurium* and the ATCC 14468 strain of *M. smegmatis* were kindly supplied by Dr. T. Mori and Dr. K. Shoji[3] of the Research Institute for Microbial Diseases, Osaka University, Osaka. A colony mass of *M. lepraemurium* was transplanted on 1% Ogawa's yolk medium (11-14), supplemented with hemin as reported by Mori (9). After six weeks cultivation at 35°C, the cells grown *in vitro* were collected into centrifuge tubes and then washed thoroughly with water by centrifugation. *M. smegmatis* was grown on the surface of Sauton medium at 35°C for five days, then harvested and washed well with water.

Preparation of crude extracts. The cells of *M. lepraemurium* and *M. smegmatis*, respectively, were ground in a mortar for 45 minutes at 4°C with the same weight of quartz sand as wet weight of bacilli, then suspended in ten volumes of 0.1 M phosphate buffer (pH 7.2). The suspension was centrifuged at 10,000 × g for 40 minutes and the supernatant thus obtained was used as a crude extract of each mycobacterium. Contents of protein in these extracts were measured by the method of Lowry *et al* (5).

Enzyme assays. Acetyl CoA synthetase (acetate: CoA ligase, adenosine monophosphate [AMP], E. C. 6. 2. 1. 1) and acyl CoA synthetases (acid: CoA ligase, AMP, E. C. 6. 2. 1. 2 and 6. 2. 1. 3) were assayed by a method partially modified from that of Samuel and Ailhaud (15). The reaction mixture contained in a final volume of 0.4 ml, 60 μmol Trihydroxymethylaminomethane (Tris)-HCl buffer (pH 7.5), 5 μmol $MgCl_2$, 5 μmol adenosine triphosphate (ATP), 1.25 μmol Coenzyme A (CoA), 1 μmol dithiothreitol (DTT), 70 μmol hydroxylamine, 0.5 μmol ^{14}C-labeled fatty acid (0.6 Ci/mol) and 1.5 mg protein from the bacillary extract. After incubation at 37°C for 15 minutes, the reaction was stopped by acidifying with 1.0 ml 2% perchloric acid, then the excess fatty acid was eliminated by washing with diethyl ether (with six 5 ml portions of ether to eliminate acetic acid in the assay of acetyl CoA synthetase, with four 5 ml portions of ether to eliminate free fatty acids in case of acyl CoA synthetase). A half milliliter of the aqueous phase was pipetted into a counting vial, mixed with 10 ml Insta-Gel (a liquid scintillation cocktail for aqueous solutions., Packard Co. Ltd.), then counted with Packard's liquid scintillation counter, Model 3385. Acetyl CoA carboxylase (acetyl CoA: carbon-dioxide ligase, adenosine diphosphate [ADP], E. C. 6. 4. 1. 2) was assayed by counting radio-activity in malonyl CoA formed from $NaH^{14}CO_3$ and acetyl CoA according to the method of Erfle (2). Assay of *de novo* fatty acid synthetase was carried out by the counting of radioactivity in fatty acids produced from 2-^{14}C-malonyl CoA and acetyl CoA according to the method of Vance *et al* (16), except for addition of 2.6-di-o-methyl

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β-cylcodextrin, one of the special stimulators for mycobacterial fatty acid synthetase ([6]), in place of mycobacterial polysaccharides ([16]), in the reaction mixture. Acetyl CoA dependent acyl CoA elongation was assayed by using a method partially modified from that of Hinsch and Seubert ([3]). The reaction mixture contained in a final volume of 1.0 ml, 100 μmol N-trihydroxymethyl-2-aminoethane sulfonic acid (TES)-KOH buffer (pH 5.6), 1 μmol DTT, 0.5 μmol reduced nicotinamide adenine dinucleotide (NADH), 0.06 μmol acyl CoA (C_4-C_{18}) as shown in Table 3, 0.15 μmol 1-^{14}C-acetyl CoA (0.4 Ci/mol) and 1 mg protein from the bacillary crude extract. The reaction mixture was incubated at 37°C for ten minutes, then 0.3 ml 50% KOH solution was added to it. The mixture was saponified for 30 minutes in a boiling water bath followed by acidification with 0.5 ml of 6 N HCl. The acidified mixture was extracted three times with *n*-pentane (acetic acid is not extracted with *n*-pentane under these conditions). Radioactivity in the *n*-pentane extract thus obtained was counted by the liquid scintillation counter.

Identification of fatty acids produced by acetyl CoA dependent acyl CoA elongation system. In order to identify fatty acids produced by the acetyl CoA dependent acyl CoA elongation system, a three times larger scale of reaction mixture than that of the ordinary assay was used. The *n*-pentane extract obtained in this case was evaporated to dryness, then the residual radioactive fatty acid mixture was methylated with 15% (W/V) BF_3 in methanolic solution ([7]). Identification of newly formed individual fatty acids by the elongation system was carried out by radiogaschromatography, using Packard's proportional counter, Model 894 connected to Packard's gas liquid chromatographic apparatus. Model 8067.

Chemicals. Sodium 2-^{14}C-acetate, sodium 2-^{14}C-propionate, sodium 1-^{14}C-butyrate, sodium 1-^{14}C-octanoate, 1-^{14}C-palmitic acid, sodium ^{14}C-bicarbonate and 1-^{14}C-acetyl CoA were purchased from the Radiochemical Center, Amersham. 2-^{14}C-malonyl CoA was purchased from New England Nuclear, Boston. Acetyl CoA, propionyl CoA, butyryl CoA, octanoyl CoA, decanoyl CoA, lauryl CoA, palmitoyl CoA and stearoyl CoA were purchased from P. L. Biochemicals, Milwaukee. 2,6-di-0-methyl β-cyclodextrin was a generous gift from Dr. A. Kawaguchi, Institute of Applied Microbiology, University of Tokyo. All other chemicals were reagent grade from commercial sources.

RESULTS

Acetyl CoA synthetase and acyl CoA synthetase activities. As shown in Table 1, it was found that the specific activity of acetyl CoA synthetase in the extract of *M. lepraemurium* was found to be about fivefold lower than that of *M. smegmatis.* Acyl CoA synthetase activities of *M. lepraemurium* for fatty acids with various chain lengths (C_3-C_{16}) were compared also with those of *M. smegmatis,* as shown in Table 1.

Acetyl CoA carboxylase activities. As shown in Table 2, activity of acetyl CoA car-

TABLE 1. *Specific activity of acetyl CoA synthetase and acyl CoA synthetase in crude extracts of* M. lepraemurium *and* M. smegmatis.

Substrate	Specific activity of acetyl CoA synthetase and acyl CoA synthetase (nmol/mg protein/min at 37°C)	
	M. lepraemurium	*M. smegmatis*
2-^{14}C-Acetate	1.87	10.93
2-^{14}C-Propionate	0.20	3.47
1-^{14}C-Butyrate	0.33	0.66
1-^{14}C-Octanoate	0.13	0.66
1-^{14}C-Palmitate	3.47	14.93

TABLE 2. *Specific activity of acetyl CoA carboxylase and* de novo *fatty acid synthetase in crude extracts of* M. lepraemurium *and* M. smegmatis.

Specific activity	*M. lepraemurium*	*M. smegmatis*
Acetyl CoA carboxylase (nmol/mg prot./min at 39°C)	undetectable	7.20
De novo fatty acid synthetase (nmol malonyl CoA/mg prot./min at 37°C)	trace (less than 0.10)	1.16

TABLE 3. *Specific activity of acetyl CoA dependent acyl CoA elongation system in crude extracts of* M. lepraemurium *and* M. smegmatis.

Starting acyl CoA	Specific activity (nmol acetyl CoA/mg prot./min at 37°C)	
	M. lepraemurium	*M. smegmatis*
Butyryl CoA	0.15	0.02
Octanoyl CoA	0.90	0.67
Decanoyl CoA	0.76	0.76
Lauroyl CoA	0.72	0.27
Palmitoyl CoA	0.42	0.13
Stearoyl CoA	0.29	0.09

boxylase in the extract of *M. lepraemurium* could not be detected, under the conditions used, whereas in the activity of this enzyme in the extract of *M. smegmatis* it was found that 7.20 nmol $NaH^{14}CO_3$ reacted to form malonyl CoA per mg protein per minute at 30°C.

De novo fatty acid synthetase activities. As shown in Table 2, activity of *de novo* fatty acid synthetase in the extract of *M. lepraemurium* was too weak to measure precisely whereas this activity in the extract of *M. smegmatis* was found to be 1.16 nmol malonyl CoA incorporated into fatty acids per mg protein per minute at 37°C.

Acetyl CoA dependent acyl CoA elongation. There has been good evidence that *Mycobacterium tuberculosis* (H_{37}Ra) retains an avidin-insensitive acetyl CoA dependent acyl CoA elongating system ([17]). For ascertaining whether a similar system is also capable of existence in *M. lepraemurium* as well as *M. smegmatis*, enzyme activities participating in acetyl CoA dependent acyl CoA elongation in these two mycobacterial extracts were investigated. In consequence, this system could be detected in both mycobacterial extracts, and specificity of these elongation systems for various acyl CoA (C_4-C_{18}) were found to be similar to each other as shown in Table 3. These elongation systems were clearly distinguishable from *de novo* fatty acid synthesis because the following features could clearly discriminate the former from the latter: 1) an avidin-insensitivity (even 100 μg/ml avidin in the incubation medium was almost insensitive) for the elongation systems; 2) the specificity of elongation for various chain lengths' acyl CoA (as shown in Table 3, the most available acyl CoA to the elongation system was octanoyl CoA or decanoyl CoA); 3) no requirement of reduced nicotinamide adenine dinucleotide phosphate (NADPH) but an absolute requirement of NADH for the elongation systems; 4) optimum 5.6 pH for the elongation systems [it is 7.0 for the *de novo* fatty acid synthetase of *M. smegmatis* ([16])]. The products formed in the reaction mixture for the acetyl CoA dependent acyl CoA elongation system in the extract of *M. lepraemurium* were identified by radiogaschromatography with authentic samples. As shown in Figure 1, radioactive lauric and myristic acids could be identified

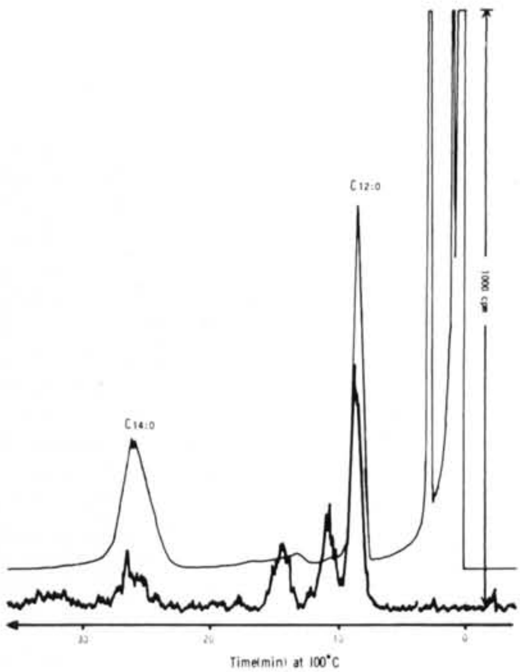

FIG. 1. Radiogaschromatographic analysis of the reaction products obtained by elongations of decanoyl CoA with 1-^{14}C-acetyl CoA. The assay system was as described in Materials and Methods. An aliquot of authentic methyl-octanoate, -decanoate, -laurate and -myristate were added to the radioactive sample and the mixture was chromatographed under an isothermal condition on a 100 cm column of Thermon 1000 (5%) (Wakojunyaku Co. Ltd., Japan) on chromosorb W (100-120 mesh). The fine and the thick lines in the figure indicate gaschromatographical and radioactivity-scanning curves, respectively, and both lines were recorded simultaneously.

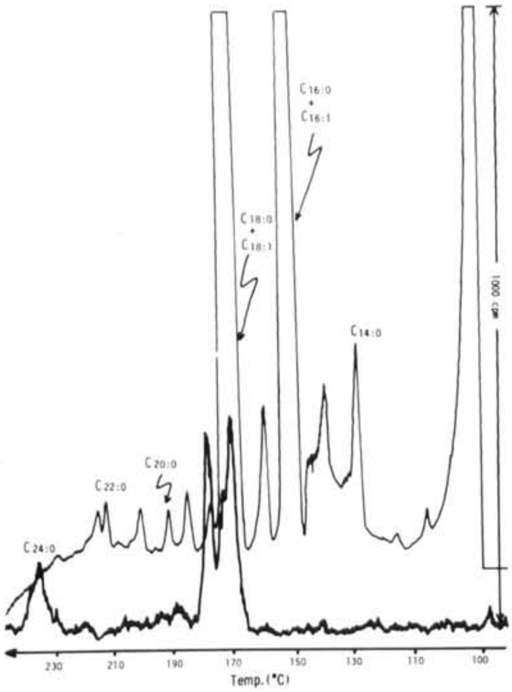

FIG. 2. Radiogaschromatographic analysis of the reaction products obtained by elongation of palmitoyl CoA with 1-^{14}C-acetyl CoA. An aliquot of lipid carrier (methyl esters of fatty acid mixture extracted from *M. smegmatis*) was added to the radioactive sample and the mixture was chromatographed under temperature-programmed conditions (5°C/minute) on the same column as Figure 1.

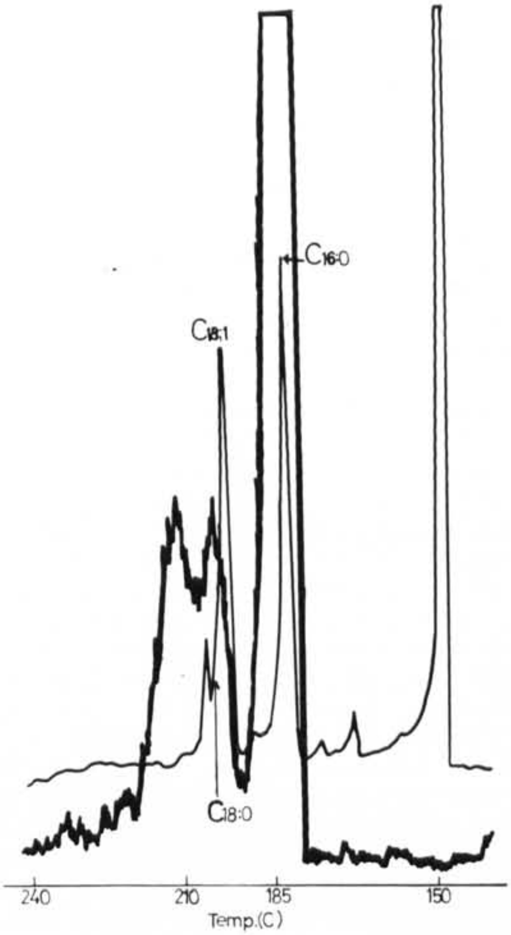

FIG. 3. Radiogaschromatographic analysis of fatty acids formed by incubation of *M. lepraemurium* with 1-^{14}C-palmitic acid. The incubation mixture contained in a final volume of 5 ml: 300 μmol phosphate buffer (pH 7.2), 5 μmol 1-^{14}C-palmitic acid (0.5 Ci/mol), 25 mg beef serum albumin (Fraction V) and 80 mg wet weight of cells of *M. lepraemurium*. After shaking the mixture at 37°C for one hour, cells were isolated from the medium by filtration, washed thoroughly with water, then saponified at 85°C for four hours with 6 ml 5% KOH in 50% ethyl alcohol. After acidifying the medium, fatty acids were extracted with diethyl ether, then methylated as described in Materials and Methods. An aliquot of the radioactive sample was chromatographed without the lipid carrier under temperature-programmed conditions (5°C/minute) on a 100 cm column of dexsil 300 (10%) (Applied Science Laboratories, U.S.A.) on chromosorb W (100-120 mesh).

in the reaction mixture, including 1-^{14}C-acetyl CoA and decanoyl CoA as substrates. Two other radioactive peaks near that of lauric acid in Figure 1 were presumed to be some intermediates occurring during the elongation of decanoyl CoA but not yet identified definitely. In addition, as shown in Figure 2, radioactive stearic acid as well as lignoceric acid could be detected in the reaction mixture for the elongation system in the extract of *M. lepraemurium,* containing 1-^{14}C-acetyl CoA and palmitoyl CoA as substrates. Other highly radioactive peaks near that of stearic acid in Figure 2 are not yet identified. Products from the acetyl CoA dependent acyl CoA elongation system in the extract of *M. smegmatis* were also identified by radiogaschromatography and it was found that the main products were similar to those in the case of *M. lepraemurium.* In attempts to elucidate a physiologic role for the acetyl CoA dependent acyl CoA elongation system in *M. lepraemurium,* its intact cells were incubated with 1-^{14}C-palmitic acid, from which elongated fatty acids during the incubation were followed by radiogaschromatography.

In consequence, a newly formed radioactive stearic acid was clearly detected as shown in Figure 3. Other radioactive peaks near that of stearic acid in Figure 3 are not yet identified.

DISCUSSION

In comparing the specific activity of enzymes pertaining to the biosynthesis of fatty acids in crude extracts of *M. lepraemurium* (an extremely slow grower) and of *M. smegmatis* (a rapid grower), it was found that these enzymes could be classified into three groups with respect to differences in their activity between these two mycobacteria. That is: 1) enzymes whose activities differ conspicuously from each other (acetyl CoA carboxylase and *de novo* fatty acid synthetase); 2) enzymes whose activities are close enough (enzymes pertaining to the acetyl CoA dependent acyl CoA elongation system); and 3) enzymes whose activities differ moderately (acetyl CoA synthetase and acyl CoA synthetase).

Acetyl CoA carboxylase and *de novo* fatty acid synthetase, belonging to the first group described, are both fundamental enzymes for biosynthesis of fatty acids among diverse living organisms. The activity of *de novo* fatty acid synthetase in a crude extract of *M. smegmatis*[4] was reported by Bloch's group ([9]), that is 1.1 nmol malonyl CoA incorporated in fatty acids per mg protein per minute at 37°C which is almost identical with that in our case (Table 2). On the other hand, the activity of acetyl CoA carboxylase in a crude extract of *M. smegmatis*[4] was also reported by Erfle ([2]), that is 26.3 nmol $KHCO_3$ incorporated in malonyl CoA per mg protein per minute at 30°C, which is about 3.5-fold higher than that in our case (Table 2). Though the reason for this discrepancy is unclear, it seems highly improbable that the activity of acetyl CoA carboxylase as well as *de novo* fatty acid synthetase in the extract of *M. lepraemurium* were missed or too little evaluated due to technical errors on our part. The reason why activities of these two enzymes in the extract of *M. lepraemurium* are so tenuous is unclear at present. Recently, it was reported that the cellular content of acetyl CoA carboxylase in *Saccharomyces cerevisiae* is reduced by the addition of long chain fatty acids to the culture medium ([4]). In addition, it has also been reported that endogenous fatty acid biosynthesis in two yeast species, *S. cerevisiae* and *Candida lipolytica* is completely repressed by the addition of long chain fatty acids to the growth medium ([8]). Though the possibility of similar phenomena could be inferred in the case of the cultivation of *M. lepraemurium* on Ogawa's yolk medium, it is very hard to evaluate this possibility because growth media other than Ogawa's are unknown up to the present.

Acetyl CoA dependent acyl CoA elongation system could be detected in *M. lepraemurium* and *M. smegmatis* for the first time. The general enzymatic properties of this system in these two mycobacteria appeared similar to each other. Further study on enzymatic properties of this system in *M. smegmatis* are being carried out in our laboratory. There seems to be good evidence that this system may be fairly common among mycobacteria, because a similar system has also been found in *M. tuberculosis* $H_{37}Ra$ ([17]).

From the relatively high activity of the acetyl CoA dependent acyl CoA elongation system in the extract of *M. lepraemurium* as shown in Table 3 as well as the evidence concerning elongation of palmitic acid occurring *in vivo* as shown in Figure 3, it seems reasonable to assume that the acetyl CoA dependent acyl CoA elongation system has a physiologic function in synthesizing long chain fatty acids in *M. lepraemurium.* On the other hand, it may be imagined that *de novo* synthesis of fatty acids has little significance for supplying fatty acids to *M. lepraemurium,* from the results shown in Table 2. Such low activity of *de novo* synthesis of fatty acids as in *M. lepraemurium* has never been reported among any other cultivable mycobacteria. These findings suggest that the extremely low activity of *de novo* synthesis of fatty acids in *M. lepraemurium* may cause a severely limited supply of fatty acids which are necessary in profusion at the moment of cell multiplication, to this microorganism. It is obvious that other factors such as the peculiar electron transfer system of *M. lepraemurium* as reported by Mori ([9, 10]) may also account for the extremely sluggish growth of this mycobacterium.

[4] *Mycobacterium phlei,* ATCC 356 used in these studies has recently been corrected to be a strain of *M. smegmatis* by the original authors ([1]).

SUMMARY

In comparing the specific activity of enzymes pertaining to the biosynthesis of fatty acids in crude extracts of cultivated *M. lepraemurium* and *M. smegmatis*, it was found that:

1. The activity of acetyl CoA carboxylase of the former organism was undetectable and that of *de novo* fatty acid synthetase was too weak to measure exactly, under the condition used, whereas both activities of the latter organism were comparable to those already reported by other authors.
2. The activity of acetyl CoA dependent acyl CoA elongation system of *M. lepraemurium* was relatively high and close to that of *M. smegmatis*.
3. The activities of acetyl CoA synthetase and acyl CoA synthetase of *M. lepraemurium* were moderately lower than those of *M. smegmatis*.

The relation between this peculiar fatty acid synthesizing enzyme system of *M. lepraemurium* and its extremely sluggish growth is discussed.

RESUMEN

Cuando se comparó la actividad específica de las enzimas que intervienen en la biosíntesis de los ácidos grasos en los extractos crudos de *M. lepraemurium* y *M. smegmatis* cultivados *in vitro*, se encontró lo siguiente: (1) En el primer microorganismo no se pudo detectar actividad de acetil CoA carboxilasa y la actividad *de novo* de la sintetasa de los ácidos grasos resultó muy débil para poder medirse con precisión. En el segundo microorganismo ambas actividades resultaron comparables a las publicadas anteriormente por otros autores. (2) En el *M. lepraemurium* la actividad del sistema de alargamiento de la acil CoA dependiente de la acetil CoA estuvo relativamente elevada y cercana a la encontrada en el *M. smegmatis*. (3) En el *M. lepraemurium*, las actividades de acetil CoA sintetasa y acil CoA sintetasa estuvieron ligeramente por abajo de las encontradas en el *M. smegmatis*.

Se discute la relación entre este peculiar sistema enzimático para la biosíntesis de los ácidos grasos en el *M. lepraemurium* y su crecimiento extremadamente lento.

RÉSUMÉ

Dans des extraits bruts de *M. lepraemurium* et de *M. smegmatis* en cultures, on a comparé l'activité spécifique d'enzymes impliqués dans la biosynthèse d'acides gras. Les observations suivantes ont été faites:

1. Il a été impossible de déceler l'activité de l'acétyl CoA carboxylase de *M. lepraemurium*. L'activité de la synthétase d'acides gras nouvellement formée était trop faible pour être mesurée de manière exacte dans les conditions de travail. Par contre, l'activité de ces enzymes chez *M. smegmatis* était comparable à celle rapportée antérieurement par d'autres auteurs.
2. L'activité du système d'élongation de l'acyl CoA dépendant de l'acétyl CoA de *M. lepraemurium* était relativement élevée, et proche de celle constaté pour *M. smegmatis*.
3. L'activité de l'acétyl CoA synthétase, de même que l'activité de l'acyl CoA synthétase de *M. lepraemurium*, étaient modérément plus faibles que les activités constatées pour ces enzymes chez *M. smegmatis*.

On discute la relation entre ces observations concernant le système enzymatique de *M. lepraemurium* pour la synthèse des acides gras, et la croissance extrêmement lente et laborieuse de ce bacille.

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REFERENCES

1. BERGERON, R., MACHIDA, Y. and BLOCH, K. Complex formation between mycobacterial polysaccharides or cyclodextrins and palmitoyl coenzyme A. J. Biol. Chem. **250** (1975) 1223-1230.
2. ERFLE, J. D. Acetyl CoA and propionyl CoA carboxylation by *Mycobacterium phlei*. Partial purification and some properties of the enzyme. Biochim. Biophys. Acta **316** (1973) 143-155.
3. HINSCH, W. and SEUBERT, W. On the mechanism of malonyl CoA-independent fatty acid synthesis. Characterization of the mitochondrial chain-elongating system of rat liver and pig kidney cortex. Eur. J. Biochem. **53** (1975) 437-447.
4. KAMIRYO, T., PARTHASARATHY, S. and NUMA, S. Evidence that acyl coenzyme A synthetase activity is required for repression of yeast acetyl coenzyme A carboxylase by exogenous fatty acids. Proc. Natl. Acad. Sci. **73** (1976) 386-390.
5. LOWRY, O. H., ROSENROUGH, M. J., FARR, A. L. and RANDALL, R. J. Protein measure-

ment with the Folin phenol reagent. J. Biol. Chem. 193 (1951) 265-275.

6. MACHIDA, Y., BERGERON, R., FLICK, P. and BLOCH, K. Effects of cyclodextrins on fatty acid synthesis. J. Biol. Chem. **248** (1973) 6246-6247.
7. METCALFE, L. D. and SCHMITZ, A. A. The rapid preparation of fatty acid esters for gas-chromatographic analysis. Anal. Chem. **33** (1961) 363-364.
8. MEYER, K. H. and SCHWEIZER, E. Control of fatty acid synthetase levels by exogenous long-chain fatty acids in the yeasts *Candida lipolytica* and *Saccharomyces cerevisiae*. Eur. J. Biochem. **65** (1976) 317-324.
9. MORI, T. Biochemical properties of cultivated *Mycobacterium lepraemurium*. Int. J. Lepr. **43** (1975) 210-217.
10. MORI, T., KOHSAKA, K. and DOHMAE, K. Terminal electron transport system of *Mycobacterium lepraemurium*. Int. J. Lepr. **39** (1971) 813-828.
11. OGAWA, T. and MOTOMURA, K. Studies on *Mycobacterium lepraemurium*. I. Attempts to cultivate *M. lepraemurium*. Lepro **38** (1969) 246-254.
12. OGAWA, T. and MOTOMURA, K. Studies on murine leprosy bacillus. II. Further characteristics of slow growing organisms isolated from experimental mice with the Hawaiian strain and an investigation of the 1% egg yolk medium. Lepro **40** (1971) 8-15.
13. OGAWA, T. and MOTOMURA, K. Studies on murine leprosy bacillus. III. Effect of treatment in the isolation culture on the invasion of saprophyte and growth of the supposed Hawaiian strain of *Mycobacterium lepraemurium*. Lepro **40** (1971) 149-155.
14. OGAWA, T. and MOTOMURA, K. Studies on murine leprosy bacillus. IV. Attempt to cultivate *in vitro* the Hawaiian strain of *Mycobacterium lepraemurium*. Further results on primary isolation, subcultivations and reproduction test of the disease in mice using "the slow growing acid-fast organisms, supposed murine leprosy bacillus." Lepro **40** (1971) 199-207.
15. SAMUEL, D. and AILHAUD, G. Comparative aspects of fatty acid activation in *Escherichia coli* and *Clostridium butyricum*. FEBS Lett. **2** (1969) 213-216.
16. VANCE, D. E., MITSUHASHI, O. and BLOCH, K. Purification and properties of the fatty acid synthetase from *Mycobacterium phlei*. J. Biol. Chem. **248** (1973) 2303-2309.
17. WANG, L., KUSAKA, T. and GOLDMAN, D. S. Elongation of fatty acids in *Mycobacterium tuberculosis*. J. Bacteriol. **101** (1970) 781-785.