Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 100 MARCH 1959 No. 3

SECTION MEETINGS

CLEVELAND, O.	
Western Reserve University	February 19, 1959
WISCONSIN	
University of Wisconsin	December 18, 1958
MICHIGAN	
Wayne State University	January 15, 1959

Growth Promoting Activity of Butterfat Acids for *Lactobacillus casei* 280-16A.* (24652)

MERRILL N. CAMIEN AND MAX S. DUNN
Chemical Laboratory, University of California, Los Angeles

Lactobacillus casei 280-16A is a D-α-hydroxy acid requiring mutant strain derived from *L. casei* 7469(1,2).† Hydrolysates of butterfat and wool wax as well as any one of a number of synthetic D- or DL-α-hydroxy fatty acids support growth of this organism (3). Saponified wool wax contains D-α-hydroxy fatty acids(4,5) which account for its growth-promoting activity. Butterfat hydrolysates, on the other hand, are not known to contain D-α-hydroxy fatty acids. Isolation and identification of the growth promoting butterfat acids were therefore attempted as described here.

Methods. Saponified butterfat was prepared and assayed essentially as described previously(3). Amphetamine salts of the butterfat acids‡ were fractionated by a series of three 100-transfer distributions between ethyl acetate and 70% ethylene glycol (by volume) in water. Growth promoting potencies and relative concentrations of solutions from the countercurrent apparatus were determined by microbiological assays and optical density (at 260 mμ) readings, respectively. Amphetamine was removed from the final microbiologically active fraction by making the aqueous phase alkaline with sodium hydroxide and extracting the mixture with several portions of ether. The residual solution was acidified with hydrochloric acid, and its content of fatty acid was taken into ether. The ether solution was evaporated, and the fatty acid was crystallized from ethanol-water.

Results. The growth promoting fraction appeared to be nearly homogeneous after the

* Paper 127, was aided by grants from U.S.P.H.S., Nat. Multiple Sclerosis Soc., and Univ. of California. The authors are indebted to Evelyn Brown and Tibor Batonay for technical assistance and to Haj Uyehara for infrared spectrography.

† Essential characteristics of strain 280-16A are the same as those originally described for strain 280-16(2,3).

‡ Prepared by mixing equivalent quantities of amphetamine (generously donated by Smith, Kline and French Labs) and butterfat acids. Amphetamine salts rather than free amino acids were employed because of convenience with which their relative concentrations may be determined photometrically.

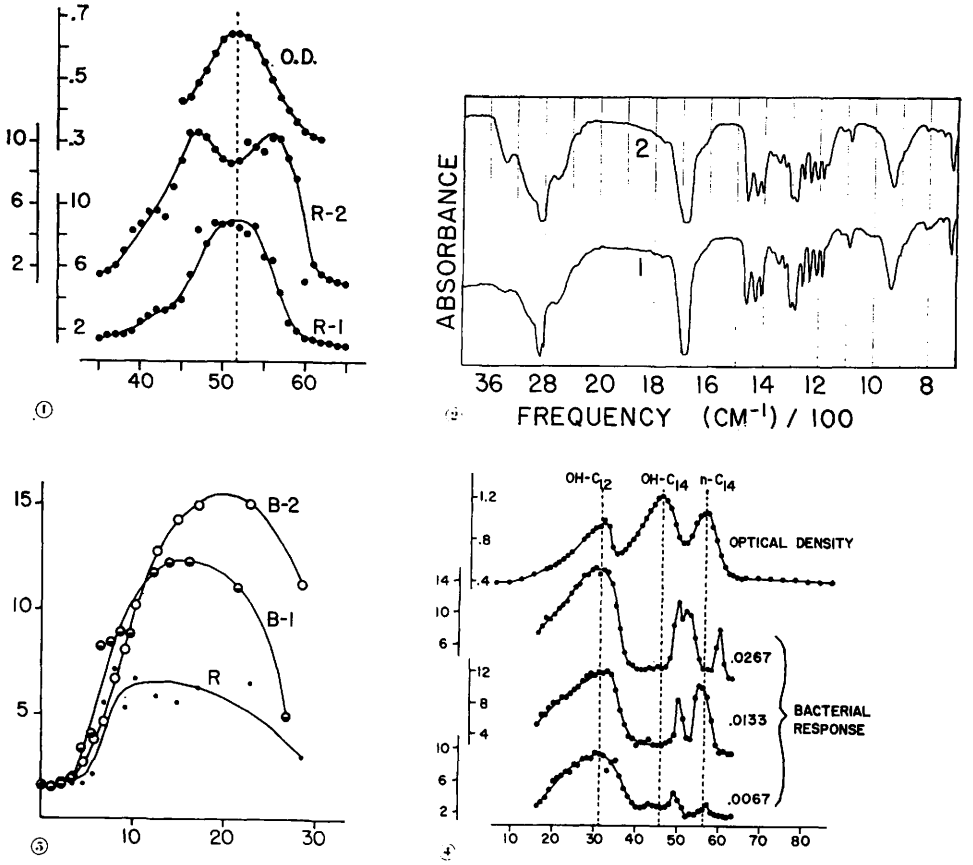

FIG. 1. Distribution of microbiologically active butterfat acid (amphetamine salt) after 100 transfers in ethyl acetate/70% ethylene glycol (by volume in water). Values on the horizontal represent the countercurrent cell numbers. Values on the upper vertical scale (curve O.D.) represent optical density (260 mμ) of upper phase solution. Values on lower 2 vertical scales (curves R-1 and R-2) represent microbiological response (ml of 0.01 N NaOH required to titrate each ml of assay culture) to 13.3 μl and 26.6 μl, respectively, of upper phase solution/ml of assay medium.

FIG. 2. Infrared spectra of the microbiologically active butterfat acid (curve 1) and purified myristic acid (curve 2). Potassium bromide pellet preparations were employed.

FIG. 3. Response of *L. casei* 280-16A to the butterfat acid (curve B-1), the residue (m. 48-51°) from crystallization of butterfat acid (curve B-2), and a reference sample of purified myristic acid (curve R). Values on horizontal scale represent the test concentrations in μg/ml. Values on vertical scale represent response in terms of ml of 0.01 N sodium hydroxide required to titrate each ml of assay culture.

FIG. 4. Distribution of an artificial mixture of DL-α-hydroxylauric acid (OH-C_{12}), DL-α-hydroxymyristic acid (OH-C_{14}), and myristic acid (n-C_{14}) (amphetamine salts) after 100 transfers in ethyl acetate/70% ethylene glycol (by volume in water). Coordinates are same as in Fig. 1. Numbers shown on bacterial response curves indicate that .0067, .0133, and .0267 ml of upper phase solution were tested/ml of assay medium.

third countercurrent distribution, and the combined contents of cells 46-56 (Fig. 1) yielded what appeared to be essentially a single fatty acid. Approximately 86% of the acid was recovered as crystalline material melting at 52.6-53.2°, and the remainder as a semi-crystalline residue melting at 48-51°. Purified myristic acid (Eastman Kodak Co. white label product), when recrystallized from ethanol-water, melted in the same range (52.6-53.2°)§ as the isolated material, and its melting point was not depressed by mixing it with the latter substance. The infrared spectrum of the recrystallized myristic acid was

§ Highly purified myristic acid melts at 54.4° according to Privett, *et al.*(6).

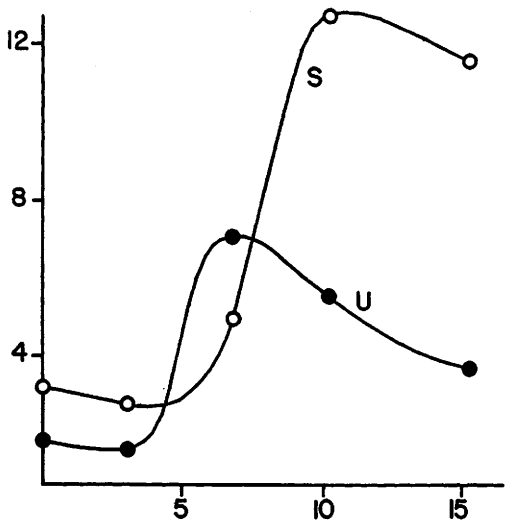

FIG. 5. Response of *L. casei* 280-16A to myristic acid in the unsupplemented assay medium (curve U) and in the same medium supplemented with 1.6 μg of DL-α-hydroxymyristic acid/ml. Coordinates are same as in Fig. 3.

essentially identical with that of the isolated butterfat acid (Fig. 2), however the latter product differed from the reference material by exhibiting pronounced growth promoting activity (Fig. 3).

These results suggested that the isolated material was essentially myristic acid but that traces of a contaminating acid accounted for its growth promoting activity. Such a contaminant would need either to possess an extremely high intrinsic growth promoting activity or to be capable of potentiating a latent growth promoting effect of myristic acid. The latter view was apparently substantiated by discovery of an analogous potentiating effect of α-hydroxymyristic acid when a artificial mixture of this substance with myristic acid and α-hydroxylauric acid was partially separated in a test of the resolving power of the countercurrent distribution system (Fig. 4). It may be seen (Fig. 4) that whereas α-hydroxymyristic acid was in itself without growth promoting activity,‖ mixtures of it with myristic acid (overlapping distributions, Fig. 4) definitely promoted growth. The potentiating effect of α-hydroxymyristic acid was also apparent when tests of myristic acid activity were made in the test medium with and without a supplement of α-hydroxymyristic acid (Fig. 5). Inhibitory effects of myristic acid at elevated concentrations have been reported(7) and are evident from the data shown in Fig. 3 and 5. These inhibitory effects account for the dip in bacterial response corresponding to peak acid concentrations in Fig. 1.

Discussion. It may be inferred from these results that the growth promoting activity of saponified butterfat for *L. casei* 280-16A is due principally to myristic acid but depends also on potentiating effects of some other acid (or acids). α-Hydroxymyristic acid is an effective potentiating substance but has a considerably lower partition ratio than myristic acid (Fig. 4) and hence could not account for the symmetrical correlation between growth response and relative concentrations shown in Fig. 1. A monohydroxypalmitic acid, on the other hand, might be expected to have nearly the same partition ratio as myristic acid,¶ and it is of interest, therefore, that such an acid has been isolated from butterfat by Bosworth and Helz(8). Whether or not the monohydroxypalmitic acid of Bosworth and Helz has a potentiating effect similar to that of α-hydroxymyristic acid has not been determined.

Summary. A fatty acid which promoted growth of *L. casei* 280-16A was isolated from saponified butterfat and identified as myristic acid together with traces of an unidentified contaminant. The contaminant was apparently necessary to potentiate the growth pro-

‖ An irregular growth promoting activity has been reported for DL-α-hydroxymyristic acid(3), however analysis of the product (eq. wt. found 242.5 calculated 244.4) suggested contamination with myristic acid, which (it is now apparent) would result in a growth promoting mixture.

¶ Increase in partition ratio of a 14-carbon fatty acid effected by removing a hydroxy group (compare α-hydroxymyristic acid and myristic acid, Fig. 4) is apparently approximately equal to the decrease in partition ratio effected by removing 2 methylene groups (compare α-hydroxymyristic acid and α-hydroxylauric acid, Fig. 4). Little or no net change in partition ratio would be expected, therefore, upon removal of both a hydroxyl group and 2 methylene groups, as in going from a monohydroxypalmitic acid to myristic acid.

moting activity of myristic acid since purified commercial myristic acid (recrystallized) was essentially inactive. Activity of the latter could be potentiated by traces of α-hydroxymyristic acid, but this substance did not appear to be present in the isolated material.

1. Camien, M. N., Dunn, M. S., *Arch. Biochem. Biophys.*, 1956, v60, 452.
2. ———, *J. Biol. Chem.*, 1953, v201, 621.
3. ———, *ibid.*, 1954, v211, 593.
4. Horn, D. H. S., Hougen, F. W., von Rudolff, E., Sutton, D. A., *J. Chem. Soc.*, 1954, 177.
5. Horn, D. H. S., Pretorius, Y. Y., *ibid.*, 1954, 1460.
6. Privett, O. S., Breault, E., Covell, J. B., Norcia, L. N., Lundberg, W. O., *J. Am. Chem. Soc.*, 1958, v35, 366.
7. Camien, M. N., Dunn, M. S., *Arch. Biochem. Biophys.*, 1957, v70, 327.
8. Bosworth, A. W., Helz, G. E., *J. Biol. Chem.*, 1935-36, v112, 489.

Received December 29, 1958. P.S.E.B.M., 1959, v100.

Trace Metal Requirements of *Aerobacter aerogenes* for Assimilation of Molecular Nitrogen.* (24653)

R. M. PENGRA AND P. W. WILSON
Bacteriology Dept., South Dakota State College, Brookings, South Dakota, and University of Wisconsin, Madison, Wis.

One of the surprising developments in biological nitrogen fixation during the past few years has been the discovery that many well-known bacteria previously unsuspected to possess the ability to use molecular nitrogen may do so when cultivated under appropriate conditions(1). These discoveries provide new tools for investigating the biochemistry of nitrogen fixation—studies that previously have been largely confined to the traditional agents of fixation: the azotobacter, the clostridia and the symbiotic system. For example, in spite of research extending over a quarter century, doubt still exists regarding the role of minerals (specifically, calcium, molybdenum and iron) in biological nitrogen fixation(2,3). Perhaps, some of the current disagreements in this respect can be resolved if attention were directed to the requirements of the new agents rather than concentration on the old. With this in mind, we have investigated the response of *Aerobacter aerogenes* strain 5al to these 3 minerals in the fixation of N_2.

Materials and methods. The growth medium and analytical procedures were essentially those previously described(2,4) altered as indicated for specific studies. The test medium for studies on the iron requirement was the same except that demineralized water prepared by passing distilled water through a Barstead "Bantamatic" demineralizer was used in all media, and iron was added separately rather than with the molybdenum solution. Solutions of the phosphate salts were run through 1 x 10 cm columns of Dowex 50 resin charged with sodium or potassium ions; a 5% solution of the corresponding chloride salt was used to charge the columns. Before passing the phosphate solutions through, the columns were washed with demineralized water until the effluent pH was near 7. A 10% solution of sucrose was purified by passing it through a similar column charged with hydrogen ions. The following solutions were prepared: Solution A, 12.5 g Na_2HPO_4 (Mallinckrodt, A. R. grade or comparable purity for all salts) in 250 ml; B, 1.5 g KH_2PO_4 in 250 ml water; C, 10 g Bacto-sucrose in 100 ml water; D, $MgSO_4 \cdot 7H_2O$ in 250 ml slurried with several grams of Dowex 50 charged with Mg ions; E. 0.02 g, $CaCl_2$ in 10 ml water; F. 2.5 μg molybdenum/ml as Na_2MoO_4; G. 2.5 μg iron/ml as $FeCl_3$. Combination of the solutions in the following quantities made up the media in the test flasks: 6.25 ml A, 6.25 ml B, 5.0 ml C, 2.5 ml D, 1 drop E, 1.0 ml F,

* Supported in parts by grants from Atomic Energy Comm. and Research Committee of Graduate School from funds by Wis. Alumni Research Fn.