PANTETHINE-REQUIRING *BACTEROIDES*

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ABSTRACT

REEVES, RICHARD E. (Louisiana State University School of Medicine, New Orleans). Pantethine-requiring *Bacteroides*. J. Bacteriol. **85:** 1197–1201. 1963.—Growth of a culture of gram-negative streptobacilli, provisionally designated *Bacteroides symbiosus*, required a preformed source of pantetheine (pantethine or coenzyme A). Five types of organisms were isolated from the parent culture, and all exhibited a pantethine requirement. Pantothenate neither replaced nor spared the pantethine requirement of these organisms. Of the five isolated bacterial types, three were characterized by producing low, medium, and high optical densities, respectively, in a thiomalate medium. A fourth type was distinguished by cells which would not pack upon centrifugation, and a fifth by its high content of stored carbohydrate. Two of the five types seem well-suited to the growth of *Entamoeba histolytica* in the MS-F ameba-culture system. In addition to pantethine, these organisms required biotin, folic acid, pyridoxine, thiamine, and choline. A minimal defined medium was elaborated for one of the isolated bacterial types. *B. symbiosus* ATCC 12829 is proposed as a test organism for pantetheine in the presence of pantothenate.

A culture of gram-negative, anaerobic streptobacilli, provisionally designated *Bacteroides symbiosus* (Stevens, 1956), is maintained in this laboratory for use in the cultivation of *Entamoeba histolytica* (Reeves, Meleney, and Frye, 1957). In preliminary studies on the comparative metabolism of the ameba and its associate organism, evidence of heterogeneity in the bacterial culture was encountered (Bragg and Reeves, 1962). It was desired to purify the bacterial types or strains to facilitate the comparison of their metabolism with that of the ameba, and to provide a more reproducible system in which to study the protozoon.

During the isolation of five types of organisms from the parent streptobacillus culture, an attack was launched on their growth requirements. This work was pushed to conclusion using one of the newly isolated bacterial types, but the significant findings appear equally applicable to the parent culture and to the other isolated culture types.

MATERIALS AND METHODS

The parent culture was derived from "streptobacillus culture no. 2" in 1955 (Shaffer, Schuler, and Key, 1958), and it has exhibited significant changes in growth characteristics during cultivation in this laboratory. Under the designation *B. symbiosus*, it was deposited in the American Type Culture Collection in 1957, ATCC 12829. A second deposit was made in 1963 (ATCC 14940), when it was recognized that our present culture is different from an ATCC 12829 culture which was re-established in this laboratory during 1962. Sebald (1962) has recently studied eight cultures provisionally designated *B. symbiosus*, all descended from the streptobacillus isolated by Shaffer, Walton, and Frye (1948), described at the same time by Shaffer and Frye (1948). Sebald (1962) classified a recent specimen of Shaffer's streptobacillus culture no. 2 as *Fusocillus plauti*, a recent culture obtained from this laboratory (designated *B. symbiosus* LSU) as *Fusiformis biacutus* (milk noncoagulating), and the ATCC culture (12829) as *Fusocillus pedipedis*.

Media. The thiomalate medium (Reeves et al., 1957) contained (per liter): Trypticase (BBL), 20 g; glucose, 10 g; sodium chloride, 2.5 g; dipotassium hydrogen phosphate (anhydrous), 1.5 g; yeast extract (Difco), 2 g; thiomalic acid (Eastman Chemical Products, Inc., mercaptosuccinnic acid, practical grade, twice crystallized from water), 1.5 g; and sodium hydroxide to pH 7.0. The components of the casein hydrolysate medium and the minimal defined medium are listed in Table 1. Except in a few experiments when added coenzyme A was employed, all components were tubed and autoclaved together for 10 min at 121 C. Coenzyme A was sterilized by

Millipore filtration and added after autoclaving. All solutions were prepared with distilled water which had been passed through a monobed deionizing filter.

Agar deeps were prepared by adding 2% agar to Thioglycolate Medium without Dextrose, no. 136C (BBL). The inocula were 0.1-ml amounts of a 10^7 dilution of a 24-hr culture. They were added when the melted medium had cooled to about 51 C. After solidifying, the cultures were capped by 2 cm of agar medium. Several days of incubation were required for colony development. Clones were established by cutting away excess agar and withdrawing an inoculum from an isolated colony by use of a needle. Several purifications through agar deeps were usually required to establish and maintain constant type characteristics.

TABLE 1. *Composition of a casein hydrolysate medium and a minimal defined medium*

Substance	Amount in medium*
L-Cysteine·HCl·H_2O	0.79 g
L-Tryptophan	0.02 g
D-Glucose	3.0 g
Mercaptosuccinnic acid	1.5 g
Folic acid	1 mg
Pyridoxylamine·2HCl	1 mg
Pantethine	2 mg
Choline chloride	8 mg
Thiamine·HCl	0.12 mg
Biotin	0.004 mg
K_2HPO_4	5 mmoles
$MgSO_4$	0.5 mmoles
$FeSO_4$	0.05 mmoles
$CaCl_2$	0.01 mmoles

* Amounts are expressed in units per liter. For the casein hydrolysate medium, 10 g of "salt-free" casein hydrolysate (acid; Nutritional Biochemicals Corp., Cleveland, Ohio) were dissolved in 250 ml of water, adjusted to pH 2.5 with HCl, and heated to 80 to 90 C for 1 hr with 5 g of activated carbon (Darko G-60). The solution was then filtered, neutralized with sodium hydroxide, passed through a Dowex 50 (Na) column, and diluted to 500 ml. This solution represented a twofold concentration of casein hydrolysate to which the components listed in the table were added. The minimal defined medium contained, in addition to the components listed in the table, L-glutamic acid, 2.5 g; L-histidine·HCl, 0.6 g; and L-phenylalanine, 0.48 g. NaOH was added to bring both media to pH 7.0.

Growth conditions. Freshly autoclaved medium was given a 1 to 2% inoculum from a preceding 24-hr culture. (Smaller inocula produced growth, but the results were erratic owing to variability of the latent period.) Day-old cultures could be refrigerated (without freezing) for several days without diminution of their ability to serve as inocula. Prior to making quantitative tests for single growth requirements, passages were made in a medium in which the factor to be tested was growth-limiting to reduce the intra- and extracellular carry-over. All cultures were incubated at 37 C.

Optical densities of the bacterial cultures were determined in a Coleman model 6A spectrophotometer, at a wavelength of 600 mμ, versus an uninoculated medium control. Screw-cap culture tubes (13 × 100 mm), containing 5-ml portions of medium, were employed.

Carbohydrate analyses were made on the suspended bacterial cells after two saline washes. Montgomery's (1957) phenol-sulfuric acid method was used, with glucose as standard.

RESULTS

Three different growth types were immediately recognized in cultures established from single colonies in agar deeps. These types exhibited, respectively, low, intermediate, and high optical densities after 24 hr of incubation in thiomalate medium. In a fourth type, the cells failed to pack after centrifugation for 10 min at 2,000 × *g*. (Indeed, these cells failed to pack after centrifugation for 1 hr at 25,000 × *g*.) Analysis of cells from the cloned cultures for carbohydrate revealed a fifth type, high in carbohydrate.

The five bacterial types were purified by repeated passages through agar deeps until their characteristics became constant, and until a further passage yielded only a single type. The five types are provisionally designated *B. symbiosus*, types *a* through *e*, and the distinguishing characteristics of each are listed in Table 2. The vast majority of first-passage isolates from the parent culture were type *c* organisms, and from the ATCC 12829 culture only type *e* organisms were obtained. Approximately 10^9 colony-forming units per ml were produced by 24-hr cultures of all types in thiomalate medium.

Vitamin requirements. Vitamin requirements were elaborated using a type *c* organism. Serial growth was first established in a casein hydroly-

sate medium supplemented with all B vitamins, tryptophan, and a generous number of other substances. Single elimination experiments reduced the number of vitamin factors to five: biotin, thiamine, a pyridoxine factor, folic acid (or leucovorin), and pantethine (or coenzyme A). The concentrations permitting one-half maximal growth (other factors in excess) were (in μg per liter): biotin, 0.24; thiamine chloride, 6.7; folic acid, 70; pyridoxylamine dihydrochloride, 70; and pantethine, 30. Concentrations tenfold greater than these usually sufficed to give optimal growth.

All five bacterial types grew serially in the casein hydrolysate medium of Table 1. Pyridoxal or pyridoxine could be substituted for the pyridoxylamine at the same concentration level. In the casein hydrolysate medium, 24-hr growth reached approximately the same optical densities as those observed for the respective types in thiomalate medium.

Upon proceeding to a simulated casein hydrolysate prepared from 15 L-amino acids (plus cysteine and tryptophan), a sixth vitamin requirement appeared. This was satisfied by added choline chloride at the level of 8 mg per liter, but not by betaine at any tested concentration up to 10^{-3} M.

Pantethine requirement. All five types, the parent strain, and ATCC 12829 appeared to have a requirement for pantethine or coenzyme A. No serial growth was obtained in any medium lacking the preformed pantetheine factor. That 10^{-3} M pantothenate neither replaced pantethine nor spared the pantethine requirement of the ATCC 12829 organism is shown by the curve at the left of Fig. 1. In this experiment, increasing amounts of pantethine were added to two sets of media tubes; one set lacked pantothenate and the other contained 10^{-3} M pantothenate. No growth occurred in either set in the presence of exceedingly low concentrations of pantethine, and the concentration of this substance permitting one-half maximal growth (0.03 μg/ml) was the same for both sets. Entirely equivalent results were obtained with a type *c* organism. Serial cultivation in pantothenate-containing media containing growth-limiting concentrations of pantethine neither altered the pantethine requirement nor gave evidence of pantothenate-utilizing bacterial types.

TABLE 2. *Typical characteristics of Bacteroides symbiosus cultures after 24 hr of incubation in thiomalate medium*

Culture	Optical density	Bacterial carbohydrate, as glucose	Cell packing after centrifugation at 2,000 × *g*
		μg/ml	
Parent culture*	0.50	124	yes
Type *a*	0.25	162	yes
Type *b*	0.25	159†	no
Type *c*	0.50	178	yes
Type *d*	0.45	446	yes
Type *e*	0.35	93	yes

* ATCC 14940 was deposited from this culture in January 1963.

† These nonpacking cells were washed four times after centrifugation at 25,000 × *g* prior to carbohydrate analysis.

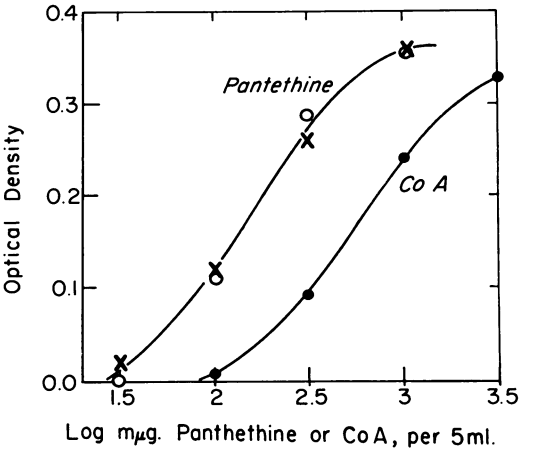

FIG. 1. *Effects of pantethine with and without added pantothenate, and of coenzyme A, upon the 24-hr growth of Bacteroides symbiosus ATCC 12829 in a casein hydrolysate medium. Open circles represent experiments with pantethine sans pantothenate; ×, pantethine plus 10^{-3} M pantothenate; closed circles, coenzyme A.*

The curve at the right of Fig. 1 shows that coenzyme A substituted for pantethine in the 12829 culture. Similar results were obtained with the parent strain and the five types. The concentration of coenzyme A permitting one-half maximal growth was approximately 0.14 μg/ml (corrected for the supplier's stated purity of the preparation). This would indicate that on a molar basis there is only a twofold difference between the activity of pantetheine in pantethine or in coenzyme A.

Amino acids. The amino acid requirements were explored with a type *c* organism. Single elimination experiments based upon the simulated casein hydrolysate medium led to the minimal defined medium of Table 1. Histidine, tryptophan, cysteine, and glutamic acid seemed to be essential; glutamic acid could not be replaced by high concentrations of alanine, glycine, or aspartic acid. Phenylalanine was stimulatory but not essential. It could be replaced by isoleucine plus other amino acids.

Types *b*, *c*, *d*, and *e* retained their original characteristics when re-established in thiomalate medium after six or seven transfers in the minimal medium. Growth in the minimal medium was considerably slower than in the other media.

Other requirements. The added iron and magnesium were essential for all of these organisms, but a requirement for added calcium ion was not established. No stimulation of growth occurred upon the addition of yeast extract ash to the minimal medium. The concentration of glucose permitting one-half maximal growth in the casein hydrolysate medium was about 0.05%.

Stimulation of growth of E. histolytica. The relative ability of the various *B. symbiosus* types to support the multiplication of amebae in the MS-F culture system is under investigation. Preliminary findings indicate little difference in this regard among types *a*, *c*, and the parent strain. The other types seem less effective, particularly type *b* which presents mechanical difficulties in the preparation of large lots of washed bacterial cells.

DISCUSSION

The organisms isolated from the *B. symbiosus* parent culture reflect the known characteristics of this culture. In unstained mounts under a phase microscope, all appear to be morphologically similar; all are pantethine-requiring. In particular, the finding of nonpacking cells recalled one occasion in which the parent culture had developed this characteristic to such an extent that the harvesting of large lots of cells was impossible. Over the years that the parent culture has been in cultivation in this laboratory, inocula were selected for high optical density growth, with the result that type *c* organisms now predominate in the parent strain. The ATCC 12829 culture was separated from the parent culture in 1957. It now appears to contain predominantly type *e* organisms. It may be that the type *c* organism is Sebald's (1962) *F. biacutus*, and the type *e* organism, her *F. pedipedis*. If Sebald's (1962) classification of provisionally designated *B. symbiosus* cultures among three species of two genera is adopted, the species designation *symbiosus* will become obsolete, since it is not possible to decide which organism Stevens (1956) used. However, classification according to the system of *Bergey's Manual* (Breed, Murray, and Smith, 1957) would, apparently, place all of these organisms in the genus *Bacteroides*.

Tamimi, Hiltbrand, and Loercher (1960) and Quinto (1962) had cultivated several *Bacteroides* on a rather complex defined medium. Their medium included all of the vitamins and amino acids of the present defined medium, except pantethine. Apparently their organisms were capable of utilizing pantothenate. Quinto (1962) stated that glutamic acid was essential for her strains, as it appears to be with the *B. symbiosus* types, but Tamimi et al. (1960) found it to be dispensable with theirs. Histidine and tryptophan seem to be essential in all instances.

With the purified casein hydrolysate medium, the vitamin requirements of all *B. symbiosus* cultures were met by added biotin, thiamine, pyridoxine, folic acid, and pantethine. Added choline was not necessary in this medium, but was required in a methionine-containing medium prepared from pure amino acids. Since neither betaine nor methionine substituted for the choline requirement, it is possible that the function of the latter is structural rather than metabolic in the formation of phospholipids.

The number of added cations required in the defined medium was surprisingly small, but there was no indication of a mineral deficiency. It has been found that the twice-crystallized thiomalic acid still carries a trace of iron, and perhaps it is also a source of other unrecognized trace minerals.

The most unusual feature of the growth requirements of these organisms was their dependence upon preformed pantetheine (pantethine or coenzyme A) in fluid media. The ATCC 12829 organism, in particular, would appear to offer promise as an assay organism for pantetheine-containing substances in the presence of pantothenic acid. Brown (1957, 1959) used *Lactobacillus helveticus* for such assays, and stated that amounts of pantothenate less than about 2 ×

10^{-7} M would not interfere with the determination of pantetheine. With the present organisms the distinction could be made much more sharply, since even 10^{-3} M pantothenate does not interfere. Craig and Snell (1951) have shown that among the lactobacilli are numerous strains which utilize pantetheine at up to a dilution 400-fold greater than pantothenate. On the other hand, with these *Bacteroides* organisms no growth was found with pantothenate in the absence of a source of pantetheine; nor, when present in 10,000-fold molar proportion, did pantothenate cause any sparing of the pantethine requirement. Brown's (1959) technique for enrichment of possible pantothenic acid-utilizing mutants or variants failed to reveal the presence of such types in either the ATCC 12829 or the parent culture.

Reeves, Meleney, and Isbell (1959) had demonstrated a pantothenate requirement for the growth of *E. histolytica* in the presence of penicillin-inhibited *B. symbiosus* cells. From evidence then at hand, it was not possible to decide whether the added pantothenate was taken up directly by the amebae or was acting via some effect upon the bacterial cells. The present finding that all types of organisms in the parent *B. symbiosus* culture are unable to use pantothenate strengthens the argument that the observed stimulation of amebic growth by pantothenate was directly due to an amebic requirement for this vitamin.

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