JOURNAL OF BACTERIOLOGY
Vol. 88, No. 4, p. 991-995 October, 1964

PRODUCTION OF EXTRACELLULAR GUANOSINE-5'-MONOPHOSPHATE BY *BACILLUS SUBTILIS*

A. L. DEMAIN, I. M. MILLER, AND D. HENDLIN

Merck Sharp & Dohme Research Laboratories, Division of Merck & Co., Inc., Rahway, New Jersey

Received for publication 2 June 1964

ABSTRACT

DEMAIN, A. L. (Merck Sharp & Dohme Research Laboratories, Rahway, N.J.), I. M. MILLER, AND D. HENDLIN. Production of extracellular guanosine-5'-monophosphate by *Bacillus subtilis*. J. Bacteriol. **88**:991-995. 1964.—Wild-type *Bacillus subtilis* colonies were found to feed purineless mutants. A strain with high feeding capacity was selected for study, with a guanineless mutant of *B. subtilis* used as the assay organism. The factor was excreted during its growth phase in a complex medium containing starch and soybean meal extract. Nutritional studies led to the development of a defined medium to be used for biochemical studies and to aid in the isolation of the factor. Starch was replaced by maltose and the soybean meal extract by Mn^{++}. Production of the factor was sensitive to the pH of the medium during growth. Practically its entire extracellular accumulation occurred before visible lysis. The factor was identified as guanosine-5'-monophosphate derived by extracellular enzymatic hydrolysis of excreted ribonucleic acid.

During studies on the nutrition of a guanineless mutant of *Bacillus subtilis*, it was observed that, when wild-type and mutant cells were present on the same agar plate, satellite growth of the mutant occurred around the wild-type colonies. The interaction was seen with various types of purineless *B. subtilis* as well as with a guanineless mutant of *Aerobacter aerogenes*. The mechanism of the feeding phenomenon was investigated with the aid of a wild-type *B. subtilis* strain which excretes high concentrations of the growth factor into liquid medium during growth. This paper describes the nutritional requirements for growth and excretion of the factor, which is shown to be guanosine-5'-monophosphate (5'-GMP). In a subsequent publication, it will be shown that the 5'-GMP arises by extracellular degradation of excreted ribonucleic acid (RNA).

MATERIALS AND METHODS

Culture. B. subtilis MB-1480 was the wild-type strain used for production of the factor. It was maintained at 4 C as a washed spore suspension.

Inoculum. The inoculum medium contained glucose (1%, w/v), salt-free, vitamin-free, acid-hydrolyzed casein (1%, w/v; Nutritional Biochemicals Corp., Cleveland, Ohio), and concentrated mineral salts mixture (10%, v/v; Demain, 1958). Deionized, charcoal-purified water was used throughout the experiments. The medium was dispensed at 20 ml in 250-ml Erlenmeyer flasks. After autoclaving, 0.05 ml of a spore suspension was added, and the flasks were incubated at 37 C for 20 hr on a rotary shaker (220 rev/min).

Production of the factor. The media were used at 20 ml per 250-ml Erlenmeyer flask. Each flask was inoculated with 0.1 ml of inoculum and incubated at 28 C for 6 days on a rotary shaker.

Assays. Growth was determined with a Bausch & Lomb Spectronic-20 colorimeter set at 660 mμ. Absorbance was measured in tubes with an inside diameter of 11.7 mm, and was converted to dry weight by means of a previously determined conversion table.

Production of ultraviolet-absorbing materials was followed by determining the absorbance of centrifuged broth at 252 mμ in a Beckman spectrophotometer (model DB). A cuvette of 1 cm light path was used. For this and other assays of excreted materials, the cells were removed by centrifugation at 25,000 × *g* for 15 min.

Quantitative estimation of the excreted material was accomplished with a tube dilution assay, with the use of the guanineless *B. subtilis* auxotroph, MB-1517 (Demain, 1963). The basal medium was the same as the inoculum medium described above, except that it was prepared at

double strength; 5 ml were added to tubes (20 by 175 mm), and water and additives were added to bring the total volume to 10 ml. After autoclaving, each tube received 0.05 ml of a spore suspension containing 10^6 to 10^7 spores per ml. The tubes were incubated at 37 C for 16 hr on a rotary shaker, and the absorbance was determined in a Spectronic-20 colorimeter at 660 mμ. This organism grows to the same extent in equimolar concentrations of guanine, guanosine, or guanosine monophosphate. The mutant also grows well on partially degraded RNA, but not on highly polymerized samples. The assay standard was disodium guanosine 5'-phosphate dihydrate, and all results were calculated as micromoles of guanines per milliliter. The term guanines is used to designate all guanine-containing compounds utilizable by the assay organism.

Sugar assays were done by a modification of the anthrone assay of Morris (1948). The changes included the use of 80% sulfuric acid and a boiling period of 10 min.

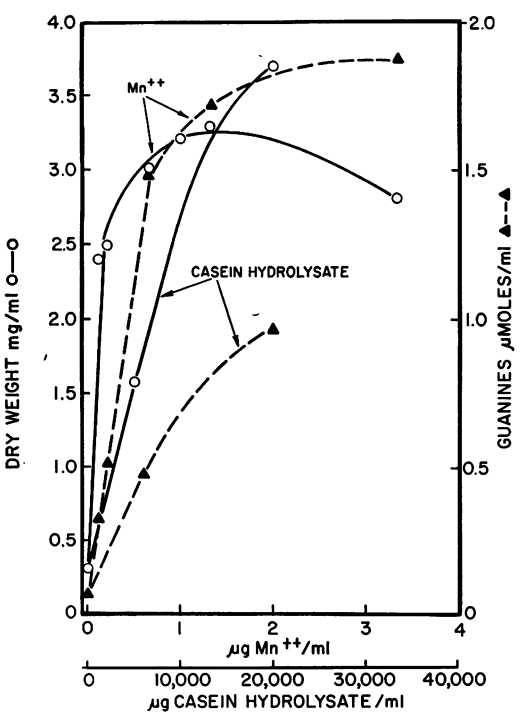

FIG. 1. *Replacement of acid-hydrolyzed casein by Mn^{++} for growth and production of guanines. Vitamin-free, salt-free, acid-hydrolyzed casein (Nutritional Biochemicals Corp., Cleveland, Ohio) was used.*

Paper chromatography. Paper chromatography was done on circular sheets of Whatman no. 1 paper in the following four systems: A, *n*-propanol–ammonium hydroxide–water (6:3:1); B, isobutyric acid–ammonium hydroxide–water (66:1:33); C, isobutyric acid–ammonium hydroxide–water (57:4:39); and D, ammonium sulfate–phosphate–*n*-propanol [30 g of $(NH_4)_2SO_4$, 50 ml of 0.1 M phosphate buffer at pH 7.0, and 1 ml of *n*-propanol]. After development and drying, the intensities of the bands were estimated by examination with an ultraviolet light source. In one experiment to be described, chromatography was also done in the descending direction on rectangular sheets.

Spot tests. The cysteine-sulfuric acid reagent (Buchanan, 1951) was used to detect deoxyribonucleosides and deoxyribonucleotides on paper. A periodate-benzidine spray modified from Cifonelli and Smith (1954) by A. C. Page, Jr. (*personal communication*) was used to differentiate 5'-ribonucleotides (positive) from 2'- and 3'-ribonucleotides (negative) and from deoxyribonucleotides (negative).

RESULTS

Nutritional studies. Of a number of wild-type strains of *B. subtilis*, strain MB-1480 was found to excrete the highest concentration of the factor allowing growth of the guanineless mutant, *B. subtilis* MB-1517. These preliminary experiments were done in a complex turbid medium containing soluble starch, sodium citrate, soybean meal extract, $(NH_4)_2HPO_4$, and other mineral salts (Nomura, Hosoda, and Yoshikawa, 1958). Nutritional studies were conducted to develop a clear, defined medium for use in biochemical studies and to aid in isolation of the factor.

Of a number of carbon sources tested as replacements for starch, maltose was best. With the disaccharide as the major carbon source, attempts were made to eliminate or replace the soybean meal extract. Despite the presence of ammonium phosphate in the medium, the removal of the extract eliminated growth. Addition of ammonium sulfate or various mixtures of amino acids failed to support growth. Whereas certain brands of casein hydrolysate were similarly inactive as a replacement for soybean meal extract, others promoted growth and production.

A third group gave rise to growth but not to elaboration of guanines. After a series of experiments which need not be described here, it was found that Mn^{++} fully replaced the active casein hydrolysates. The divalent cation was 50,000 times as active as the hydrolysate on a weight basis (Fig. 1). The Mn^{++} requirement for production of guanines was four times that required for growth. Half-maximal production occurred at 0.4 μg/ml. This higher requirement for production of guanines is apparently the reason that certain brands of casein hydrolysate supported growth but not excretion of guanines.

The discovery of the Mn^{++} requirement, and data from nutritional experiments concerning optimal concentrations of other medium ingredients, resulted in the defined medium shown in Table 1. This medium was used in the subsequent studies.

Time course of production. Production of the growth factor was studied as a function of time in the chemically defined medium (Fig. 2).

TABLE 1. *Defined medium for growth and excretion of guanines**

Component	Amt
	g/liter
Maltose†	80.0
Sodium citrate·$2H_2O$	12.0
$(NH_4)_2HPO_4$	20.0
KCl	1.5
$MgSO_4 \cdot 7H_2O$	0.5
$CaCl_2 \cdot 2H_2O$	0.15
$CuSO_4 \cdot 5H_2O$	0.15
$MnSO_4 \cdot 4H_2O$	0.015

* The pH was 7.0.
† Autoclaved separately.

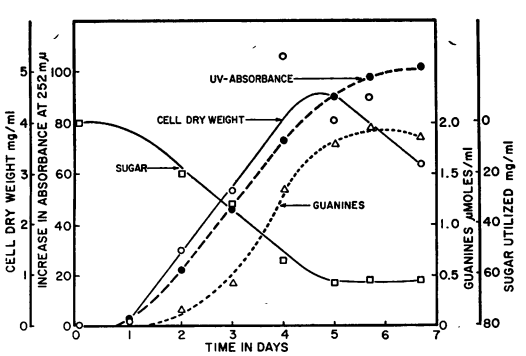

FIG. 2. *Time course of changes during growth of Bacillus subtilis MB-1480.*

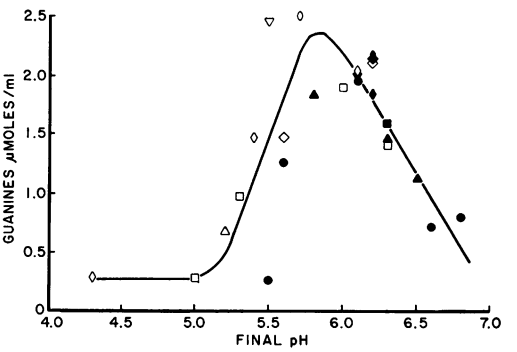

FIG. 3. *Production of guanines as a function of final pH (6 days). The pH was varied by adjustment before autoclaving and by inclusion of 0.25% L-aspartic acid. Each of the seven experiments represented in this figure are indicated by a different symbol. Open symbols indicate the absence and closed symbols indicate the presence of aspartic acid in the medium.*

After a lag period of about 1 day, growth became visible and continued until the fifth day. Of interest was the linear kinetics of growth. From the fifth to seventh days, lysis was noted as a decrease in absorbance. Sugar utilization was also rapid during the phase of growth and ceased at 5 days. Excretion of material absorbing at 252 mμ followed growth closely, and approximately 90% of the ultraviolet-absorbing material was elaborated before lysis was evident. The appearance of bioactive guanines lagged behind the elaboration of ultraviolet-absorbing materials, but practically all of the bioactivity was found in the medium prior to the onset of gross lysis.

Effect of pH. During a study on the effect of additives, it was found that the addition of certain amino acids (e.g., aspartic acid) stimulated the production of guanines. This was found later to be an indirect effect by which the amino acid prevented a drop in pH during the course of growth. In a series of experiments, the pH during growth was varied by adjusting the pH before autoclaving and by growing the cells in the presence or absence of 0.25% L-aspartic acid. Figure 3 is a summary of the results obtained in seven separate experiments. It can be seen that optimal conditions for production of guanines are those that resulted in a final pH of 5.6 to 6.2. These facts were taken into consideration in developing the medium shown in Table 1. Visible effects on growth were not evident in these experiments, suggesting that

production of guanines is more sensitive to pH changes than is growth.

Identity of 5'-GMP. To determine the identity of the material which supported growth of *B. subtilis* MB-1517, an 8% perchloric acid extract of whole broth was examined by circular paper chromatography in system A after removal of perchloric acid by neutralization with potassium hydroxide and centrifugation. Four ultraviolet-absorbing bands were seen at R_F 0.16, 0.22, 0.44, and 0.57. The two slowest bands were the most prominent, and had mobilities expected of nucleotides in this solvent system. The chromatogram was cut into ten sections, each of which was eluted into assay medium, autoclaved, and inoculated with the assay organism. Only the R_F 0.16 band showed bioactivity.

A large quantity of this band was obtained by paper chromatography on several sheets, elution, and lyophilization. Spot tests of this material on paper showed a positive reaction with periodate-benzidine and a negative test with cysteine-sulfuric acid. These tests eliminated from consideration guanine, 2'-GMP, 3'-GMP, deoxyguanosine, and all isomers of deoxy guanosine monophosphate. The lyophilized preparation was chromatographed in systems A, B, and C. In each case, an ultraviolet-absorbing band migrated with authentic 5'-GMP in cochromatography. That the lyophilized material retained bioactivity was shown by chromatographing it in the descending direction in system A, cutting the sheet into sections, eluting the sections, and assaying each one with MB-1517. Guide strips on the side of the strip were used to identify the location of various authentic guanine-containing compounds. The results in Fig. 4 indicated that the active material in the lyophilized preparation was 5'-GMP.

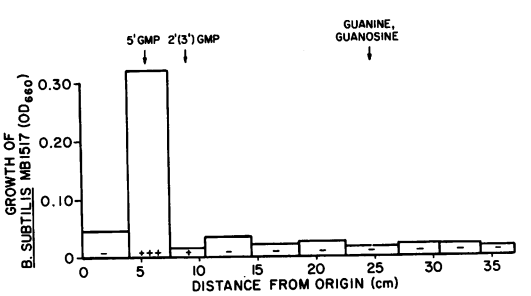

FIG. 4. *Bioassay of sections from paper chromatogram of lyophilized preparation. The intensity of ultraviolet absorption is denoted by plus and minus marks within the bars. The migrations of authentic compounds are shown at the top.*

Further confirmation was obtained when it was found that the bioactive factor was retained by Dowex 1-X2 (Cl^-) anion exchange resin, was not eluted by 0.005 M HCl, but was eluted by 0.1 M HCl. The resin eluate showed two bands by paper chromatography in solvents A and D. In each case, the slower band cochromatographed with authentic 5'-GMP. The resin eluate showed a spot by treatment with periodate-benzidine but no reaction with cysteine-H_2SO_4.

DISCUSSION

The present work demonstrates that wild-type *B. subtilis* is able to accumulate extracellular 5'-GMP and thus feed a guanineless mutant. Details on the mechanism involved will be presented in a subsequent paper. Suffice it to say that intact nucleic acids are excreted, and the RNA is degraded extracellularly to nucleoside 5'-monophosphates. The Mn^{++} requirement for growth and production of 5'-GMP is of interest in this connection. Since Mg^{++} was present in the medium at a concentration 500-fold greater than the concentration of Mn^{++} required for half-maximal growth, it is evident that a specific Mn^{++} requirement exists. That four times as much Mn was needed for guanine accumulation than for growth suggests that the additional Mn was used for synthesis of the RNA which is ultimately excreted. This concept is in agreement with results from cell-free studies on RNA polymerase directed by deoxyribonucleic acid (Furth, Hurwitz, and Goldmann, 1961; Fox and Weiss, 1964) and by RNA (Chung, Mahler, and Enrione, 1960; Fox et al., 1964). In these studies, Mn^{++} was the most active divalent metal ion for RNA synthesis, and was active under certain conditions where Mg was totally inactive.

ACKNOWLEDGMENTS

We wish to thank Joanne F. Fabian and Livia Kelemen for skillful technical assistance.

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