MICROBIAL PRODUCTION OF VITAMIN B_{12} ANTIMETABOLITES. III
COMPOUND 102804 FROM *BACILLUS CEREUS*

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A basic compound with empirical formula $C_{12}H_{16}N_2O_5$ was isolated from *Bacillus cereus* 102804 fermentations of a soybean meal - glucose medium. The inhibitory activity of compound 102804 on growth of Gram-positive and Gram-negative bacteria growing in a chemically defined medium was reversed by vitamin B_{12}, by L-methionine, and by D-methionine. It has no inhibitory activity for *Escherichia coli* (Davis 113–3) when grown in media containing L-methionine.

The biosynthesis of 102804 coincides with the sporulation of the *B. cereus*, and the compound is not produced in the absence of sporulation.

A search for microorganisms producing substances inhibiting vitamin B_{12}-stimulated growth of *Escherichia coli* (Davis 113–3) resulted in selection of a strain of *Bacillus cereus* which had this capability. The vitamin B_{12} antimetabolite is produced by this culture (designated as 102804 in our collection) when grown under several conditions, and its production seems to coincide with sporulation of the bacillus. We wish to summarize in this communication our studies on the effect of fermentation conditions on production of compound 102804, and some of the chemical and biological attributes of this vitamin B_{12}-antimetabolite.

Materials and Methods

Isolation of culture 102804

A soil sample collected in Madison was diluted with sterile water and plated on nutrient agar. Discrete colonies were transferred to tubes of soybean meal - glucose - $CaCO_3$ medium and placed on a shaker at 30°C. After several days' growth aliquots of the fermented medium were centrifuged and the supernatant liquid tested for ability to inhibit growth of *E. coli* (Davis 113–3) growing in an agar diffusion bioassay in the DAVIS - MINGIOLI[1] medium (with added vitamin B_{12}), and to inhibit the growth of *Escherichia coli* B(ATCC 11303) (growing in nutrient agar) and *Staphylococcus aureus* FDA 209P (ATCC 6538) (growing in nutrient agar). The supernatant solution from culture 102804 was found to inhibit the *E. coli* (Davis 113–3) and not the other two organisms under the test conditions, and the inhibition was competitively reversed by vitamin B_{12} in an agar diffusion bioassay.[2] Culture 102804 was maintained for further study by periodic transfers on agar slants and by storage of cell suspensions in liquid nitrogen.

Identification of culture 102804

Culture 102804 was grown in a variety of media mentioned in BERGEY's manual[3] and compared with cultures of *Bacillus cereus* (ATCC 14579), *Bacillus mycoides* (ATCC 6462), and *Bacillus subtilis* (ATCC 6633) grown under the same conditions. Culture 102804 is closely related to *B. cereus* on the basis of: Colonial morphology on glucose-containing agar; Gram staining characteristics; shape of sporangia; vegetative cell and spore size; acid production when grown on glucose and glycerol containing agars, and no acid production when grown on mannitol and xylose containing agars; a positive VOGES-PROSKAUER test and a positive test for reduction of nitrate to nitrite.

Bioassay of vitamin B_{12} antimetabolite activity from *B. cereus* 102804

Samples from media fermented by *B. cereus* 102804 were assayed for the presence of vitamin B_{12} antagonists by an agar diffusion method and a turbidimetric method. The agar diffusion assay involved placing 12.7 mm paper discs dipped in the test solutions on the surface of agar plates seeded with *E. coli* (Davis 113-3) and noting the diameter of the inhibition zones after incubation of the agar plates at 37°C for 18 hours. (The plates were prepared as previously described[4] with the addition of 20 mcg of cyanocobalamin to 200 ml of the DAVIS-MINGIOLI medium). The activity which showed a 20-mm diameter inhibition zone was defined as 1 unit. The slope of the dose response curve was usually about 3 mm.

The turbidimetric assay used was a modification of that of BURKHOLDER[5] using 25×150 mm test tubes (containing 10 ml of medium) as culture vessels and incubation at 37°C for 17 hours.

The ability of amino acids and vitamin B_{12} to reverse the growth inhibitory effects of compound 102804 on *E. coli* (Davis 113-3) and other organisms was measured by a modification of the SMITH[2] agar diffusion method.

The inhibitory activity of 102804 on growth of bacteria and yeast was determined by agar-diffusion bioassay using both the DAVIS-MINGIOLI medium[1] and Difco antibiotic assay medium No. 11 (which contains beef extract, yeast extract, casitone, peptone, and dextrose).

Tissue culture cytotoxicity tests

Cytotoxicity of 102804 preparations was determined by the standard procedures of the National Cancer Institute[6] using KB cells, P-388 cells, and L-1210 cells.

Isolation of compound 102804 from fermented media

Ten liters of fermented soybean meal - glucose medium with a potency of 25 units/ml were adjusted to pH 3.0 by addition of 6 N HCl and the mixture was then centrifuged. The supernatant solution was then passed through a column (8.0×25 cm) of Amberlite IR-120 resin (50~100 mesh, H^+ cycle). The antimetabolite was eluted with 0.6 M aqueous pyridine. After removing the pyridine from the 'bioactive fractions' *in vacuo*, the solution was lyophilized. The 12.6 g of crude powder thus obtained had a potency of 22 units per mg. It was then dissolved in 100 ml of pH 3.5 0.06 M pyridine-HCl buffer and the solution applied to a column (4.5×80 cm) of Dowex 50W-X4 resin (100~200 mesh), buffered at pH 3.5 with the pyridine HCl buffer. The column was eluted with 20 liters of this pH 3.5 buffer and the 'bioactive fractions' pooled and adjusted to pH 8.0 using 1 N NaOH. The pyridine was removed *in vacuo* and the solution passed through a 4.0×25 cm column of Dowex 50W-X4 resin (100~200 mesh, H^+ cycle). The antimetabolite was eluted with 0.6 M pyridine and the 'bioactive fractions' pooled. The pyridine was removed by evaporation *in vacuo* and the resulting aqueous solution lyophilized; 1.05 g of a powder with potency of 160 units per mg was obtained. This was dissolved in 5 ml of distilled water and 15 ml of acetone were added; 0.90 g of crystalline material were obtained with a potency of 160 units per mg. The total recovery of bioactivity from the fermented medium was 58%.

The crystalline material gave a single spot when examined by thin-layer chromatography (using ninhydrin spray to locate the material on the TLC) with Rf 0.25 in *n*-butanol - acetic acid - water (4:1:2), and Rf 0.50 in methanol - acetone - water (2:2:1). It also gave a single spot in ionophoresis at pH 1.9 (acetic acid - formic acid buffer) with a mobility of 19 cm when the current was 85 volts per cm.

Chemical and physical analyses

The melting point was determined using a Thomas-Hoover capillary apparatus. Optical rotation was determined with a Perkin-Elmer polarimeter (model 241). IR spectra were obtained in KBr disc using a Perkin Elmer spectrophotometer (model 257). Elemental analyses were performed by the Spang Microanalytical Laboratory, Ann Arbor, Michigan.

Results and Discussion

Factors Affecting Antimetabolite Production by *B. cereus* 102804

Preliminary study showed that antimetabolite production by *B. cereus* 102804 was coincident with spore formation and not related to cell growth: Good growth was obtained when shaken flask fermen-

tations were incubated over the range 25° to 40°C, and maximum antivitamin B_{12} activity was obtained when the cultures were incubated at 30°C. The best medium for antivitamin B_{12} activity production contained 2% glucose, 1% $CaCO_3$, and 2% soybean meal (or aqueous extract of soybean meal), and the optimum aeration rate was K_d 4.0×10^{-6} g mole O_2 per ml per minute per atmosphere.

Data collected in a shaken flask fermentation of the soybean meal - glucose medium are summarized in Fig. 1. Bacterial cell growth reached a maximum after about 10 hours incubation (with a specific growth rate of 0.7 $hour^{-1}$). Sporulation and production of vitamin B_{12} antimetabolite reached their maximum after about 20 hours incubation. Continued incubation of the fermentation resulted in rapid drop in the titer of the vitamin B_{12} antimetabolite, and this inactivation was shown to be an enzymatic process.

A continuous fermentation study was carried out using a 4-liter Erlenmeyer shaken (260 rpm) flask with 4 baffles. The working volume was maintained at 500 ml using a peristaltic pump, and the pH was maintained at pH 7 by adjustment of the soybean meal extract - glucose nutrient feed. Some of the data collected are summarized in Fig. 2. We found that maximum cell productivity occurred with a dilution rate of 0.6 per hour, and maximum vitamin B_{12} antimetabolite production was obtained at a dilution rate of 0.4 per hour which practically coincided with the optimal dilution for maximum sporulation rate.

As earlier studies with spore forming anaerobes[8] had shown changes in nutritional requirements and metabolic patterns when these organisms were grown in the absence of sporulation, we examined the relationship between sporulation and production of vitamin B_{12} antimetabolite by non-sporulating cultures of *B. cereus* 102804 obtained by repeated transfer of the culture at 16-hour intervals. Some of the data collected as summarized in Fig. 3: As the number of transfers increased, the sporulation

Fig. 1. Time course study of fermentation of soybean meal - glucose by *Bacillus cereus* 102804.

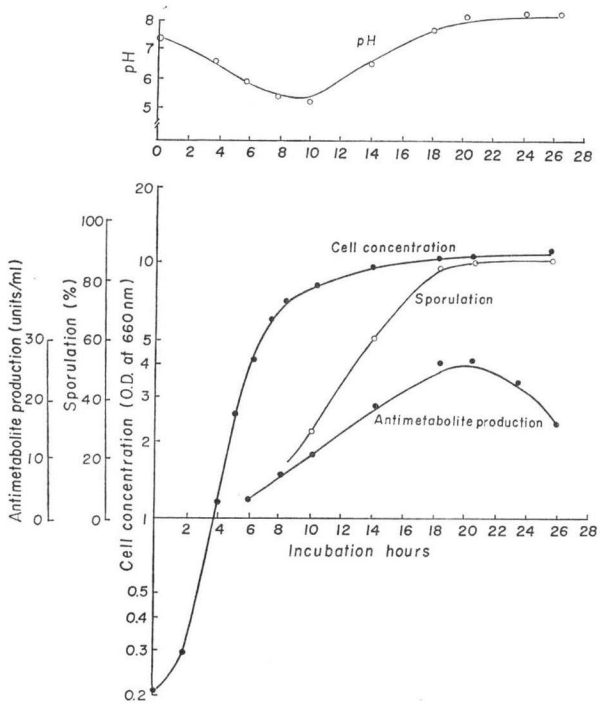


Fig. 2. Steady-state relationships in a single-stage continuous fermentation.

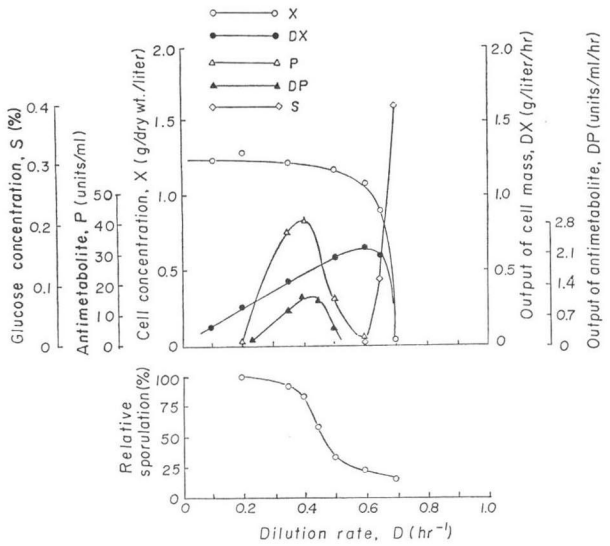


Fig. 3. Decrease of production of compound 102804 by *Bacillus cereus* 102804 upon repeated transfer without sporulation.

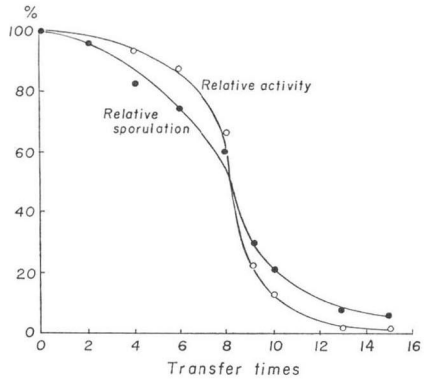
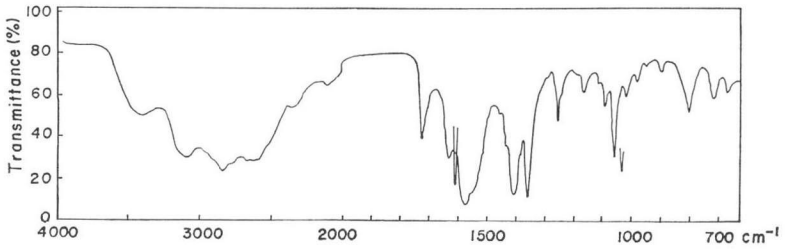


Fig. 4. Infrared spectrum of compound 102804.



rate and production of vitamin B_{12} antimetabolite gradually decreased. Both sporulation and vitamin B_{12} antimetabolite levels were less than 10% of the maximum obtained (with the first transfer) after 10 transfers even though the generation time and final cell concentration did not change during these transfers.

Table 1. Antimicrobial activity of crystalline compound 102804

Test organism	Minimal inhibitory concentration, mcg/ml	
	grown on medium A	grown on medium B
Escherichia coli (Davis 113–3)	3.2	>1,000
growth stimulated by B_{12} (25 ng/ml)		
growth stimulated by methionine (100 ng/ml)	>1,000	>1,000
Escherichia coli B (ATCC 11303)	6.2	>1,000
Pseudomonas aeruginosa (Ps 24)	50	>1,000
Staphylococcus aureus FDA 209P	0.4	>1,000
Bacillus subtilis ATCC 6633	no growth	25
Candida albicans ATCC 73481	1,000	>1,000

Medium A: K_2HPO_4, 7 g; KH_2PO_4, 3 g; $(NH_4)_2SO_4$, 1 g; Na_3 citrate, 0.5 g; $MgSO_4$, 0.1 g; glucose (sterilized separately), 10 g; distilled water q.s. 1 liter.

Medium B: Difco medium No. 11.

Fig. 5. Vitamin B_{12} reversal of compound 102804 inhibition of growth of *Escherichia coli* (Davis 113-3).

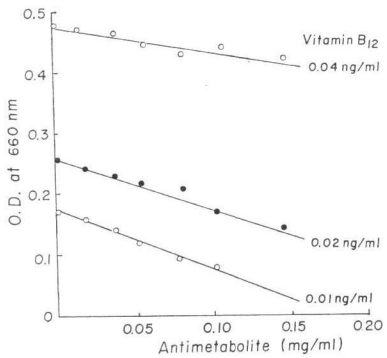
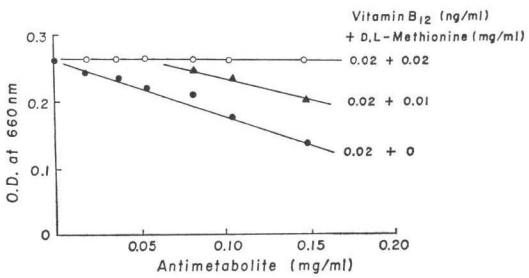


Fig. 6. D,L-Methionine reversal of compound 102804 inhibition of growth of *Escherichia coli* (Davis 113-3).



Physical and Chemical Properties of Crystalline 102804

The following data were obtained with the crystalline material:

1) m.p.: 145~147°C (dec.)
2) Rotation: $[\alpha]_D^{20}$ +16° (*c* 1, H_2O)
3) Elemental analysis: Found: C, 46.79; H, 6.69; N, 9.59
   Calculated for $C_{12}H_{16}N_2O_5 \cdot 2H_2O$: C, 47.36; H, 6.58; N, 9.21
4) Molecular weight (by high resolution mass spectroscopy): 268.27
5) U.V. absorption: 280 nm ($E_{1cm}^{1\%}$ 40); 300 nm ($E_{1cm}^{1\%}$ 35 (shoulder))
6) I.R. absorption as shown in Fig. 4

The crystalline material gave positive ninhydrin test (yellow), iodine test, and permanganate test, and negative anthrone test.

Biological Properties of Compound 102804

Crystalline 102804 inhibited the growth of both Gram-negative and Gram-positive bacteria when the organisms were grown in the chemically defined medium (see Table 1) and were not inhibited when they were grown in the Difco antibiotic assay medium No. 11. As the *E. coli* (Davis 113–3) was inhibited when growth was stimulated by vitamin B_{12} on a competitive basis (see Fig. 5) and was not inhibited when the growth was stimulated by D- or L-methionine, we conclude that the mechanism of action on *E. coli*

(Davis 113–3) involves inhibition of methylation.

The crystalline 102804 did not inhibit the growth of the KB cells, P–388 cells, and L–1210 cells when grown in the serum supplemented test media.[6]

Conclusions

These studies with *B. cereus* 102804 and earlier studies[7,8] with *B. cereus* 439 show that strains of this species can synthesize several types of compounds which are vitamin B_{12}-antimetabolites under certain test conditions.[2] These may also function as amino acid antimetabolites[7,8] and their role in the metabolism of the producing organism is at present uncertain. Their production appears to coincide with sporulation of the *B. cereus* cells and they may be an intermediate in the biosynthesis of certain amino acids and related compounds formed during sporulation.

Acknowledgements

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