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Levels of Cyclic GMP in Dormant, Germinated, and Outgrowing Spores and Growing and Sporulating Cells of *Bacillus megaterium*

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The level of cyclic GMP was less than one molecule per organism in dormant, germinated, and outgrowing spores of *Bacillus megaterium*. A significant level (~8 pmol/g, dry weight) of cyclic GMP was found in early to mid-log phase cells, but the level fell to below 0.2 pmol/g, dry weight, in late-log phase and only rose slightly to ~0.9 pmol/g, dry weight, in stationary phase. No significant amount of cyclic GMP was detected in the growth medium at any time.

The cycle of growth, sporulation, and spore germination and outgrowth in bacteria of various *Bacillus* species has been considered a model system for study of the regulation of differentiation. Considerable effort has been expended on this system in attempts to identify small molecules which might regulate parts of the overall differentiation process. These studies have shown in part: (i) that cyclic AMP (cAMP), an important regulatory molecule in many bacteria such as *Escherichia coli*, is not present in any stage of the life cycle of several *Bacillus* species (1, 6); and (ii) that cyclic GMP (cGMP) is present in growing cells of *Bacillus licheniformis* and the levels of this compound change significantly as cells go from log-phase growth to stationary phase (1). Guanylate cyclase and cGMP phosphodiesterase have also been detected in *B. licheniformis* (3, 5). Because of our interest in the regulation of spore germination and outgrowth, we were most interested in the findings on cGMP. Consequently, we decided to determine levels of cGMP throughout the differentiation cycle of *Bacillus megaterium*, with special emphasis on dormant, germinated, and outgrowing spores.

Because of the extremely low levels of cGMP which have been reported in *B. licheniformis* (1), we were concerned that centrifugation of cells before their extraction might drastically affect cGMP levels. Consequently, in our experiments we extracted small molecules from cultures of cells or spores without prior centrifugation, but rather by addition of trichloroacetic acid directly to growing cultures. This necessitated purification of any cGMP before its assay (Table 1). The procedure for cGMP purification was adapted from one previously described for cAMP purification (6) and gave recoveries of ~30% of small amounts (~10 pmol) of [^{14}C]-cGMP added to parallel cultures just after addition of trichloroacetic acid; all values reported in this communication have been corrected for the loss in cGMP during sample purification. In addition, as previously noted in determinations of cAMP in *Bacillus* species (6), we found a small but reproducible amount of material which reacted as cGMP when samples of growth medium or even water were subjected to our cGMP purification procedure. Presumably this inhibitory material arose from some contaminant in one of our reagents. This material amounted to ~0.25 pmol of cGMP per purified sample, and all values we obtained were corrected for this background value.

Dormant and 70-min germinated spores of *B. megaterium* contained a small amount of cGMP (Table 1); this material was destroyed by incubation with cyclic nucleotide phosphodiesterase (Sigma Chemical Co.). However, the significance of the small amount of cGMP in dormant and 70-min germinated spores is unclear, since the levels are significantly less than one molecule per organism (Table 1). Whether this indicates that a fraction of the population does contain a molecule or two of cGMP, or simply is due to variation in our data (see variation in Table 1), is unclear. However, the levels of cGMP in spores germinated 15 or 30 min were below the limits of detection (Table 1).

When spores were germinated at ~0.5 mg/ml in a medium which supported spore outgrowth, again any cGMP present was below the limit of detection (0.5 pmol/g), even though >80% of the outgrowing spores had reached the first cell division by the time the last sample was taken (Fig. 1a). However, log-phase growth had not yet begun, and the culture was relatively syn-

TABLE 1. *Absence of cGMP from dormant and germinated spores of B. megaterium*[a]

Spores analyzed	cGMP level (pmol/g, dry wt)
Dormant	0.4 ± 0.2[b] (0.35)[c]
Germinated[d] 15 min	<0.2 (<0.18)[c]
Germinated[d] 30 min	<0.2[e] (<0.18)[c]
Germinated[d] 70 min	0.5 ± 0.25[e] (0.45)[c]

[a] Spores of *B. megaterium* QM B1551 (originally obtained from H. S. Levinson, U.S. Army Natick Labs, Natick, Mass.) were prepared by growth at 30°C, harvested, washed, lyophilized, and stored as previously described (7). Dormant spores (1 g, dry weight) were added to 50 ml of boiling 80% 1-propanol to extract all small molecules (8). After 10 min of boiling the solution was cooled, flash evaporated, and suspended in 50 ml of cold 5% trichloroacetic acid. A small amount (10 pmol) of [^{14}C]cGMP (New England Nuclear Corp.) was added to one half of the mixture to monitor cGMP recovery during subsequent purification. After incubation of 4°C for 40 min, the mix was centrifuged (10 min, 10,000 × *g*), and the pellets were washed with 10 ml of 5% trichloroacetic acid. Both supernatant fluids were combined, and 1.5 g of acid-washed charcoal was added. After gentle swirling for 60 min at room temperature, the supernatant fluid was removed, the charcoal was washed four times with 25 ml of 1 mM HCl, and the charcoal was packed into a small column and washed with water (20 ml). Adsorbed material was eluted from the column with 40 ml of ethanol:water:ammonium hydroxide (60:90:9). The eluate was flash evaporated to dryness, dissolved in 5 ml of 50 mM sodium acetate (pH 4.0), and centrifuged (10 min, 10,000 × *g*), and the pellet was discarded. The supernatant fraction was applied to a column (1 by 10 cm) of Dowex-1 acetate and washed successively with 50 mM sodium acetate (pH 4.0) (35 ml), water (40 ml), and 2 N HCOOH (40 ml). The cGMP fraction was then eluted with 4 N HCOOH (40 ml). This eluate was flash evaporated, and the residue was dissolved in 1 ml of water and centrifuged (10 min; 10,000 × *g*), and any pellet was discarded. Aliquots of the supernatant fluid were then run on descending paper chromatography on Whatman no. 1 paper for 20 h with 1-propanol:NH_4OH:H_2O (60:30:10) as the solvent, with small amounts (<1 pmol) of [^{14}C]cGMP in adjacent tracks to serve as guides for subsequent location of the cGMP zone. The cGMP zone was located from the migration of the ^{14}C-markers, cut out, eluted, lyophilized, and dissolved in water before analysis. Overall recoveries of cGMP determined either by counting or by cGMP analyses (see below) were quite reproducible and gave a mean of 31.3% for 15 separate extractions with a standard deviation of 6.1%. All values have been corrected for cGMP loss during purification. cGMP was determined by radioimmunoassay according to the method of Steiner et al. (11) by using acetylation of samples to increase sensitivity (2). The kit supplied by New England Nuclear Corp. (catalog no. NEX-133) was used for these assays, which were carried out in duplicate with two sample concentrations and a calibration curve constructed using various amounts of cGMP (0.0025 to 0.5 pmol).

chronous (data not shown). In a second experiment in which growth was initiated from a log-phase culture, a significant level of cGMP was found in early to mid-log-phase cells (Fig. 1b). The cGMP level then fell to an extremely low value, and then rose slightly during stationary phase (Fig. 1b). In the latter experiment the evidence that the material reacting in the assay was indeed cGMP was as follows: (i) the radioimmunoassay we used has been shown to be extremely specific for cGMP (2); (ii) the material was purified with authentic cGMP through our purification scheme; and (iii) the material was destroyed (>85%) by incubation with cyclic nucleotide phosphodiesterase (Sigma). In neither of the experiments shown in Fig. 1 did we detect any cGMP (<1 fmol/ml) in the growth medium (samples taken at arrows in Fig. 1a and b; data not shown), in contrast to the finding with *Escherichia coli* (9). The results shown in Fig. 1b are similar to those obtained previously with *B. licheniformis* (1), although the levels of cGMP seen in the latter study are higher than found by us in *B. megaterium*. As noted previously (1), the levels of cGMP in *Bacillus* species appear significantly lower than do those in *E. coli* (1, 4).

The results presented in this communication confirm that cGMP is present in cells of *Bacillus* species in at least one stage of the growth cycle, although at a rather low level. However, the absence of a significant level of cGMP (≥1 molecule per organism) from dormant, germinated, and outgrowing spores and its extremely low level during the transition from log-phase growth to stationary phase make it extremely

All values were corrected for the small amount of material which reacted as cGMP from samples of water or medium subjected to the purification procedure. This was equivalent to ~0.25 pmol per purified sample, and this value was very reproducible within individual experiments.

[b] Average value from three separate experiments.

[c] Values in parentheses are calculated values of molecules of cGMP per organism. These values were calculated by using $7 \cdot 10^8$ as the number of spores per milligram, dry weight. This value was determined by plating appropriate spore dilutions on nutrient agar plates and counting the number of colonies formed.

[d] Dormant spores (1 g) were heated at 60°C at 20 mg/ml in water for 15 min. After the spores were cooled, they were germinated in 0.05 M potassium phosphate (pH 7.4) and 0.05 M glucose at 30°C and a spore concentration of 2.5 mg/ml. Greater than 95% of the spores had initiated germination by 10 min. At indicated times, a 350-ml sample was made 5% in trichloroacetic acid, divided in half, and treated as described in footnote *a*.

[e] Average value from two separate experiments.

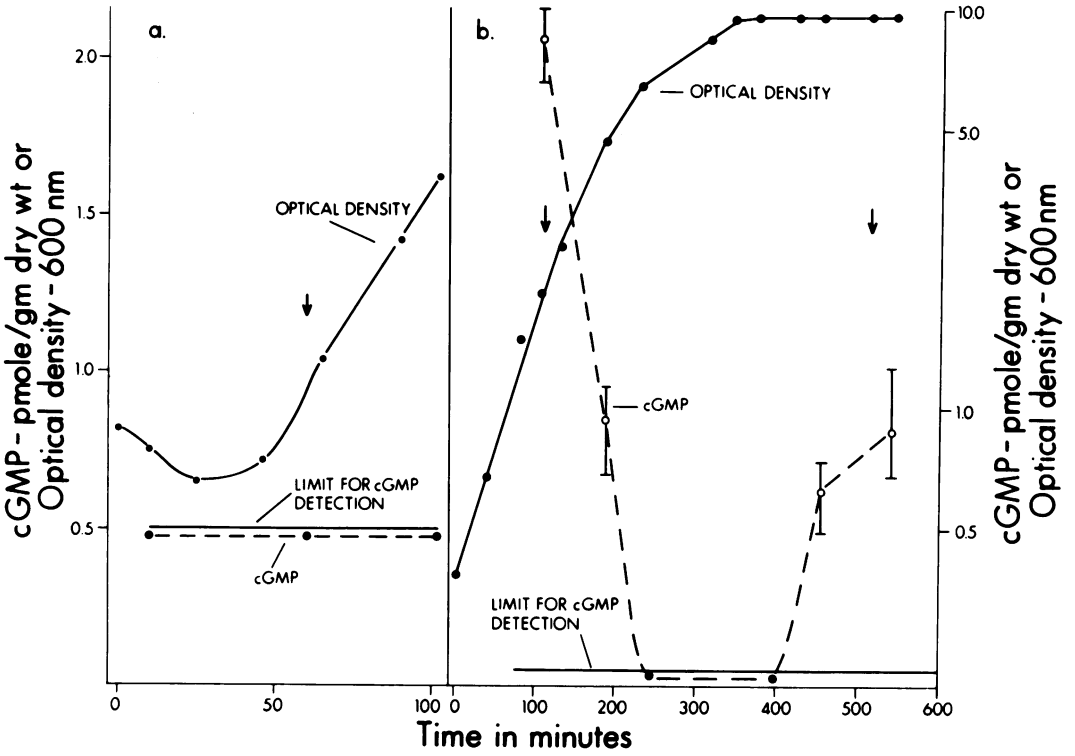

FIG. 1. *Levels of cGMP in (a) outgrowing spores and (b) growing and stationary-phase cells. (a) Spores (2 g) were heat shocked and then germinated at 5 mg/ml as described in the legend to Table 1. After 10 min the culture was centrifuged (10 min; 10,000 × g), and the pellet was washed once with ~200 ml of prewarmed (30°C) Spizizen (10) medium containing 0.1% Casamino Acids and finally suspended in 4 liters of this medium at 30°C. Samples of 350 ml were taken and treated as described in the legend to Table 1 except that separate 350-ml samples were taken for determinations of cGMP recoveries. At several points (arrows) samples (350 ml) were also taken for analysis of cGMP in the culture fluid. These samples were centrifuged, trichloroacetic acid was added to the supernatant fluid, and then the purification procedure was carried out. Approximately 80% of the spores underwent the first cell division between 70 and 90 min. (b) Cells were grown at 30°C in Spizizen medium (10) containing 0.1% Casamino Acids. Samples were taken and treated as described above. The arrows denote the times when samples were taken for analysis of cGMP in the medium. The filled circles on the cGMP curves in both (a) and (b) mean that the cGMP values from samples taken at these times were below the limits of detection. The error bars in (b) represent the variation for replicate analyses.*

unlikely that this compound is involved in regulating the differentiation processes undergone by organisms of the *Bacillus* species. What the exact function of cGMP is in *Bacillus* species, as well as other bacteria, is clearly a matter for further work.

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