INDUCTION OF SPORULATION IN BACILLUS SUBTILIS BY DECOYININE OR HADACIDIN

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SUMMARY

Bacillus subtilis, growing exponentially in the presence of rapidly metabolizable carbon, nitrogen and phosphate sources, can be induced to sporulate by the addition of decoyinine, a specific inhibitor of GMP synthesis, or of hadacidin, a specific inhibitor of AMP synthesis. Optimal sporulation is obtained at inhibitor concentrations causing only partial inhibition of growth.

INTRODUCTION

Sporulation of bacilli is normally initiated only when rapidly metabolizable carbon or nitrogen sources are replaced, at the end of growth or by cell transfer to a new medium, by slowly metabolizable compounds. This observation has led to the postulate that sporulation may be repressed by a carbon and nitrogen containing compound (1). Attempts to identify such a compound have shown that many mutations and inhibitors can prevent sporulation. Therefore, an alternative hypothesis proposed that the initiation of sporulation (asymmetric prespore septation) results from the establishment of a new biosynthetic balance between, on the one hand, a reduced rate of RNA, protein and cell wall synthesis (reduced cell expansion) and, on the other hand, a

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continued rate of membrane synthesis and the concomitant movement of the chromosome origins toward the cell poles (2). Consequently, a partial (but not complete) inhibition in the synthesis of any metabolite, which produces the proper biosynthetic balance, might induce sporulation under conditions under which it would normally not occur. We now report that limited starvation for purine nucleotides, produced by addition of decoyinine or hadacidin, induces sporulation in B. subtilis.

MATERIALS AND METHODS

Growth Conditions. Our standard sporulating strain (60015) of B. subtilis was grown overnight at 37°C. Next morning when the optical density at 600 nm (OD_{600}) had reached 0.5, 5 ml aliquots of the culture were distributed into 25 mm diameter prewarmed tubes that contained 0.1 ml of the drug used. To follow growth, 0.2 to 0.5 ml samples were diluted in S6 and the OD_{600} was measured. Doubling times were determined by the change in OD_{600} values 2-3 hours after the addition of the drug. The total viable cell titer (V) was determined by plating on tryptose blood agar base (33 g/l; Difco) plates. Spore titers (S) were measured by heating the diluted cultures for 20 min at 75°C and then plating. S6 medium contained: 100 mM potassium morpholinopropane sulfonic acid, pH 7; 5 mM potassium phosphate, pH 7; 10 mM ammonium sulfate; 1 mM $MgCl_2$, 0.7 mM $CaCl_2$; 50 μM $MnCl_2$; 5 μM $ZnCl_2$; 5 μM $FeCl_3$; 25 mg/ml L-tryptophan; and 10 mg/ml L-methionine. As the carbon source, the medium contained 1 percent glucose except for the experiments stated in Table 2.

Compounds. Decoyinine U-7984 was kindly sent to us by Dr. George W. Whitfield of The Upjohn Co., Kalamazoo, Michigan, and sodium hadacidin was kindly sent by Mr. Walter B. Gall of Merck Shark & Dohme Research Laboratories, Rahway, New Jersey.

Assays. Glucose was semi-quantitatively determined with Diastix strips from Miles Laboratories, Inc. Ammonia was assayed with Nessler's reagent obtained from Fisher Scientific Company. Phosphate was analyzed according to Chen (3). Malate was assayed according to Gutmann and Wahlefeld (4).

RESULTS

Decoyinine is a specific inhibitor of GMP synthetase (5). Figure 1A shows that increasing concentrations of decoyinine, added during exponential growth at OD_{600} = 0.5, progressively reduced the rate of growth. The titer of spores, observed 8 hours

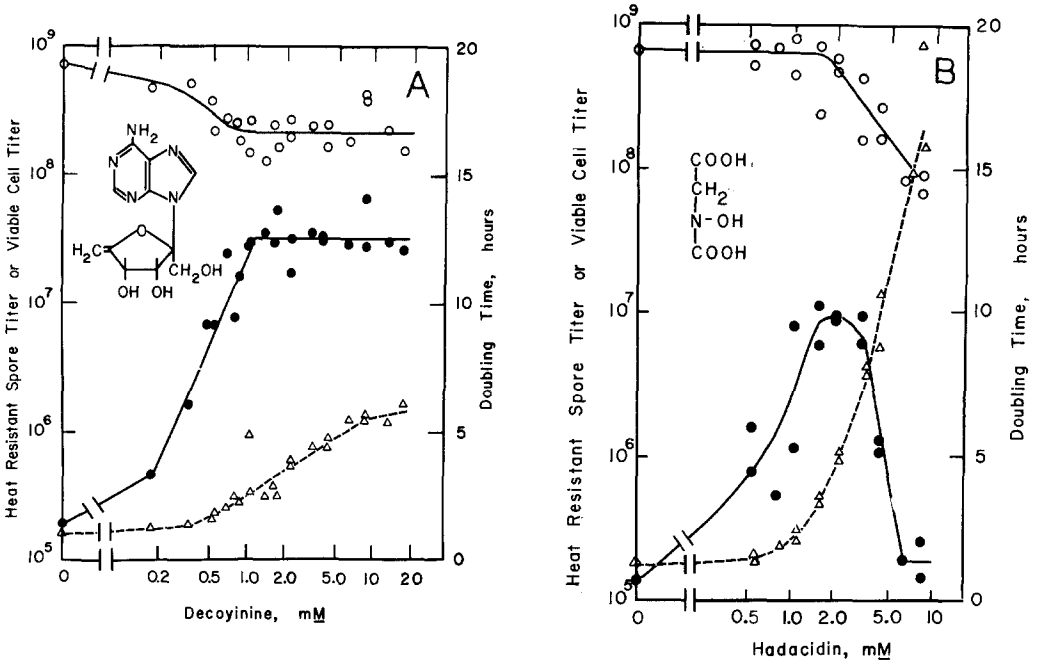

Fig. 1 Induction of sporulation by decoyinine or hadacidin. Doubling times (▲) were measured within the first 2 or 3 hours after addition of the compound during midexponential growth (OD_{600}=0.5). Eight hours after addition, the cells were plated to determine the total viable cell titer (○) and the heat resistant spore titer (●). A. decoyinine (left); B. hadacidin (right).

after decoyinine addition (t_8), also increased with increasing decoyinine concentration whereas the total viable cell titer was only marginally affected. Figure 2 shows that the sporulation frequency increased significantly 5 hr after addition of 1.8 mM decoyinine and then continued to increase to a maximum of 50 percent.

In contrast to decoyinine which inhibits GMP synthesis, hadacidin specifically inhibits adenylosuccinate synthetase and thus AMP synthesis (6). Increasing concentrations of hadacidin decreased the growth rate, but for this compound high concentrations caused almost complete cessation of growth (Fig.

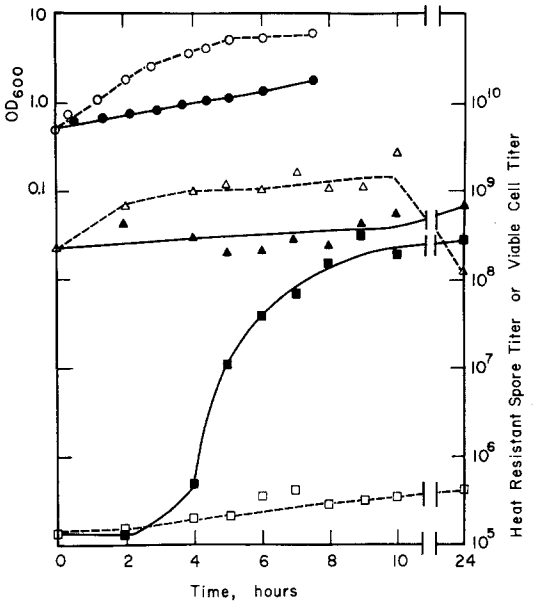

Fig. 2 Time course of growth and sporulation after decoyinine addition. Growth was followed by measuring the OD_{600} (circles). The total viable cell titer (triangles) and the heat resistant spore titer (squares) were determined by plating at different times. Without decoyinine (hollow symbols), with 1.8 mM decoyinine (full symbols).

1B). The spore titer reached a maximal value at an intermediate hadacidin concentration (about 2 mM), and the vegetative cell titer decreased at high hadacidin concentrations owing to cell lysis (decrease of OD_{600}).

To demonstrate the specificity of these effects, the inhibitors were added together with different purines and the inhibition of growth and induction of sporulation (S/V) were measured (at t_{10}). As table 1 shows, the effect of decoyinine was counteracted only by guanine whereas that of hadacidin was counteracted only by adenine.

To ascertain that the essential ingredients of the medium were not exhausted during the residual growth, the concentrations

TABLE 1. Specificity of Decoyinine and Hadacidin

Additions to S6 + 1% Glucose Medium	Doubling Time (hours)	S/V at t_{10}
none	1.4	2.5×10^{-4}
+1.8 mM decoyinine	5.7	.49
+1.8 mM decoyinine + 0.3 mM adenine	7.2	.32
+1.8 mM decoyinine + 0.3 mM guanine	1.7	2.4×10^{-3}
+1.8 mM decoyinine + 0.3 mM xanthine	6.1	.41
+1.8 mM decoyinine + 0.3 mM hypoxanthine	8.5	.71
+ 2.1 mM hadacidin	4.5	3.2×10^{-2}
+ 2.1 mM hadacidin + 0.3 mM adenine	1.7	7.3×10^{-4}
+ 2.1 mM hadacidin + 0.3 mM guanine	4.5	1.8×10^{-2}
+ 2.1 mM hadacidin + 0.3 mM xanthine	4.6	2.5×10^{-2}
+ 2.1 mM hadacidin + 0.3 mM hypoxanthine	5.7	1.7×10^{-2}

of glucose, ammonia, and phosphate were measured at t_{10}. Their concentration had decreased by less than 50 percent under concentrations of optimal sporulation. To exclude further the possibility that the inhibitors indirectly inhibit the uptake or early metabolism of glucose, decoyinine was added to cells grown in excess of other carbon sources. As shown in table 2, sporulation was also observed in the presence of malate, sorbitol, glycerol, or casein hydrolysate plus glucose. In the latter case, a relatively high sporulation frequency (20%) could be observed (at t_{10}) only if 1 mM methyl anthranilate was added together with decoyinine to prevent the immediate germination of the produced spores (7). In the case of malate, optimal sporulation was obtained at 0.45 mM decoyinine, higher concentrations allowing

TABLE 2. Induction of Sporulation in Different Media

Media	Doubling Time (hours)		S/V at T_{10} (%)	
	Decoyinine*		Decoyinine*	
	-	+	-	+
S6 + 1% glucose	1.1	3.3	.012	33
S6 + 1% sorbitol	1.6	2.8	.018	12
S6 + 1% glycerol	1.1	2.9	.0091	31
S6 + 50 mM K^+-malate	1.1	2.5	.012	13
S6 + 1% glucose + 1% vitamin-free casein hydrolysate + 1 mM methyl anthranilate	.52	2.3	.00014	13

* Decoyinine used at 1.8 mM except in media containing K-malate were decoyinine was used at 0.42 mM.

less sporulation (by t_{10}). Since malate is rapidly metabolized, its concentration was measured and found to be 12 mM at t_6 and 1 mM at t_9.

DISCUSSION

Our results demonstrate that both decoyinine and hadacidin induce a significant number of cells of *B. subtilis* to sporulate in the presence of rapidly metabolizable sources of carbon, nitrogen, and phosphate, i.e., under conditions under which the cells would normally not sporulate. Apparently, the limitation of AMP alone or of GMP alone are sufficient to allow this sporulation. This result seems to suggest that a limitation in the synthesis of some compound, such as RNA or DNA, whose synthesis requires metabolites derived from both GMP and AMP suffices to initiate sporulation. This suggestion agrees with

other results, to be reported elsewhere, in which we show that sporulation can be induced also by other inhibitors of nucleotide pathways, including 6-azauracil which inhibits UMP synthesis. However, nucleotide synthesis is known to be controlled by complex feedback interrelationships; it is therefore not excluded that the diverse inhibitors cause the decrease or increase of a single small molecular weight compound which controls sporulation. In contrast to certain sporulation mutants (blocked at stage II), the asymmetric septa produced under our conditions are not all filled with so much cell wall material that further engulfment is prevented; possibly, the nucleotide limitation exerts some additional control on wall synthesis (while this is not surprising for UMP or AMP derivatives, it is unexpected for GMP derivatives).

Hadacidin induces sporulation only if it is used at an intermediate inhibitory concentration. Decoyinine shows a similar optimum when the growth medium contains malate. These findings show that sporulation is induced when nucleotide synthesis is limited but not completely inhibited. We would like to suggest that this observation explains why only one out of many glutamine requiring and only a few of many purine requiring mutants isolated in B. megaterium can sporulate in the presence of excess glucose and ammonia (8,9): the sporulating mutants may all be leaky in the synthesis of nucleotides. In fact mutants isolated in our laboratory as being able to sporulate under these conditions were all found to be leaky purine mutants (Heinze and Freese, to be published).

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