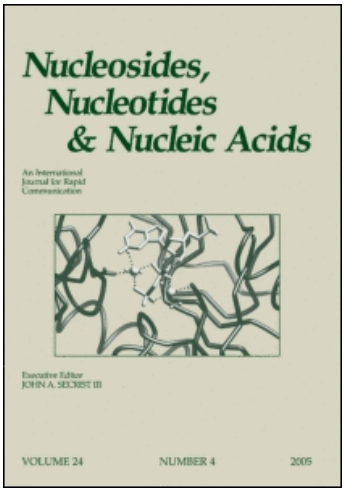

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IMPAIRMENT OF Streptomyces AND Micromonospora DIFFERENTIATION BY DNA-METHYLASE INHIBITORS

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ABSTRACT

The effect of several inhibitors of DNA methyltransferases has been tested on Streptomyces and Micromonospora cultures. Some of them (5-aza-2'-deoxycytidine, 5-azacytidine and L-ethionine), inhibited sporulation at concentrations that did not significantly affect growth.

INTRODUCTION

DNA methylation has been demonstrated to play an important role in eukaryotic gene expresion[1-3]. Inhibitors of this reaction, such as 5-azacytidine (5-AC), cycloleucine or L-ethionine have succesfully induced gene expression in different systems[2,4-7]. DNA methylation is also involved in cellular differentiation and carcinogenesis[1,2,6,8]. Methylated bases in prokaryotic DNA are typically associated with restriction-modification systems, though there are some exceptions, like the *dam* and *dcm* systems of E. coli. The *dam* methylase is involved in mismatch repair, transposition, recombination and gene expression[9-11]. Some data in Spiroplasma, Myxococcus and Bacillus also support the idea that DNA methylation could have a role in prokaryotes development[12-19]. Differences in the DNA methylation patterns

during the life cycle have been reported for the three genera, in which vegetative growth is characterized by hypomethylation compared to early stages of differentiation or sporulation[12,15,19]. Petridou and Slepecky[14] describe stimulation of microcycle sporulation in Bacillus, by S-adenosyl-L-methionine and inhibition by 5-azacytidine. These results are clearly related to phospholipid metabolism, but, as suggested by the DNA methylation changes[15], pleiotropic effects which would also affect DNA modification can not be ruled out. Some authors have suggested a relationship between differentiation and DNA methylation in actinomycetes[20-26]. Barbés and coworkers[23-24] have shown that S. antibioticus ETHZ 7451 has at least three DNA-methylase activities none of which could be assigned to any restriction-modification system; moreover, S. antibioticus DNA methylation state varies during sporulation[24]. This strain is capable of sporulating in submerged culture[27], providing a suitable system to study the effect of DNA-methylase inhibitors on physiological and morphological differentiation. Our results showed that, like in Bacillus[14,16], some of these compounds prevent differentiation.

MATERIAL AND METHODS

Strains and growth conditions. Different Streptomyces and Micromonospora strains have been used in this study, including S. antibioticus ATCC 11891, S. antibioticus ETHZ 7451, S. antibioticus ATCC 8663 (type strain), S. glaucescens ETHZ 22794, S. coelicolor A3(2) (Hopwood, wild type), S. coelicolor ETHZ A 3170, M. chalcea ATCC 12452 (type strain), M. halophytica KCCA 0125, M. melanospora KCCA 0063 and Micromonospora sp. IMET 8002. GAE, SM, SPM and GYM media have been previously described, as well as MOPSMg and MESMg buffers[27]. Solid media were prepared with 2% agar. Inhibitors were sterilized by filtration and added to the sterile media before pouring into the plates.

TABLE 1.

TYPE	COMPOUND	CONCENTRATION	MEDIA	REFERENCE
I	5-Aza-2'-deoxycytidine	50 μM[a] 1-10 μM[b]	SM	2,6
I	5-Azacytidine	0.5-2 mM[a] 0.075-5 μM[b]	SM,MOPSMg	2,6
I	5-fluoro-2'-deoxycytidine		0.5-2 mM SM	
	27			
I	Cordycepin	0.1-2 mM	SM,MESMg	29
I	L-Ethionine	1.5-6 mM	SM,MOPSMg	4,5
I	Cycloleucine	0.5-2 mM	SM	7
I	Dimethylsulfoxide	25-500 μM	SM	30
II	Adenine	1-5 mM	SM,SPM	14
II	Adenosine	1-5 mM	SM,SPM	14
II	L-Methionine	1 mM	SM	
II	S-Adenosil-methionine	1 mM	SM	17
III	Chloramphenicol	25-80 μg/ml	SM,MOPSMg	
III	Rifampicin	1-10 μg/ml	SM,MOPSMg	
III	Mitomycin C	1-5 μg/ml	SM,MOPSMg	
III	Novobiocin	5-25 μg/ml	SM,MOPSMg	

DNA-methyltransferases inhibitors and other compounds at the concentration tested and liquid media where experiments were performed. Inhibitors were also tested in solid GAE, GYM and SM at the same concentrations. Type I include methylation inhibitors, type II SAM precursors and type III antibiotics.(a) Assayed in *Streptomyces*; (b) Assayed in *Micromonospora*

For submerged cultures, spores were preincubated overnight in GYM and transferred to SM, SPM, MESMg or MOPSMg plus the tested compound (see Table 1) as previously described[27]. Inhibitors of DNA methylation, and other compounds used in submerged cultures, and the conditions tested are summarized in Table 1. S-adenosyl-L-methionine (SAM) and precursors were only tested in submerged cultures. Growth of submerged cultures was measured in up to four

replicas of each culture by optical density determination as previously described[27]. Growth of solid cultures was estimated. Sporulation was monitored by phase-contrast microscopy.

Chemicals. S-Adenosyl-L-methionine was obtained from Boehringer. All the other compounds were from Sigma.

RESULTS

Effect of DNA-methylases inhibitors on Streptomyces and Micromonospora surface cultures. Table 2 shows the effects of DNA-methylase inhibitors on actinomycetes growth and differentiation.

It should be noted that medium composition strongly influenced the inhibitory effect observed with 5-AC and L-ethionine, but 5-AC plates always sporulated after 5 days (data not shown). The behaviour of other Streptomyces and Micromonospora strains was similar, though differences on sensitivity could be observed. Micromonospora was found to be much more sensitive to 5-AC than Streptomyces, requiring concentrations 200-fold lower for inhibition (Table 2). L-ethionine impaired the differentiation process at the stage of substrate mycelium (Fig. 1C) and 5-AC allowed the development of aerial mycelium (Fig. 1B), though spore formation did not take place in the presence of either. 5-aza-2'-deoxycytidine (5-dAC) was only assayed in Micromonospora chalcea cultures; its effect was the same as with 5-AC, but lower concentrations were needed (Table 2).

Effect of DNA methylase inhibitors and S-adenosylmethionine precursors on Streptomyces antibioticus ETHZ 7451 submerged cultures. S.antibioticus sporulates in liquid SM medium after preincubation in GYM; in SPM differentiation is partially repressed[27]. The addition of inhibitors to S. antibioticus submerged cultures resulted in the same effect as in the SM, GAE or GYM solid cultures

TABLE 2

Effect of DNA-methylation inhibitors on Streptomyces (a) and Micromonospora (b) surface cultures.

INHIBITOR	CONCENTRATION	GROWTH	SPORULATION
5-azacytidine[a]	0.5 mM	++	-
5-azacytidine[b]	25 µM	++	-
5-fluoro-2'-deoxycytidine[a,b]	2 mM	++	+
L-ethionine[a,b]	1.5 mM	++	-
Cycloleucine[a,b]	2 mM	++	+
Dimethylsulfoxide[a,b]	25-500 µM	+	+

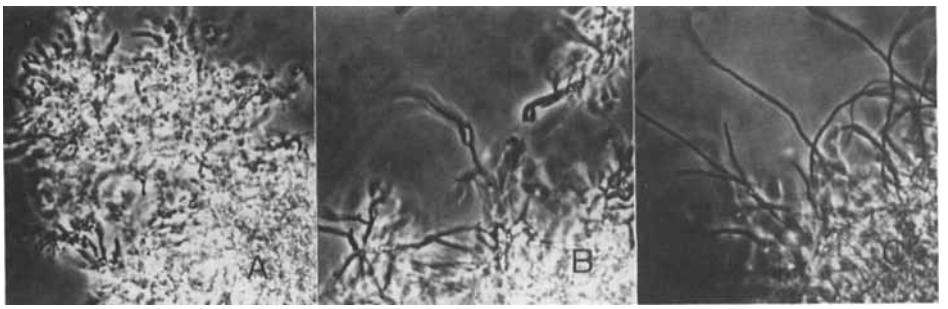

FIGURE 1.

Phase contrast microphotographs of S. antibioticus ETHZ 7451 cultures grown in SM (A) and in the presence of 0.5 mM 5-AC (B) or 1.5 mM L-ethionine (C); (x400). The two types of inhibition are shown: L-ethionine inhibition at the substrate mycelium stage (A) and 5-azaC inhibition at aerial mycelium stage(B).

(Table 3). In the case of compounds that did not impair sporulation only results at maximum concentration are given, but they are not significantly different that those for lower concentrations. Higher 5-AC concentrations partially inhibited growth, and lower concentrations of SAM had no effect on sporulation. In the case of L-ethionine, higher concentrations enhanced growth even more, and lower concentrations allowed differentiation.

As observed on surface cultures, sporulation impairment was exerted at different morphological stages by L-ethionine and 5-AC or 5-dAC. The Streptomyces and Micromonospora life cycles start with spore germination and the formation of substrate (vegetative) hypha, which are typically long and thin. Under particular circumstances, a new morphological type of hypha appears: shorter and thicker aerial (reproductive) hypha. These reproductive hypha undergo septation and, finally, spores mature and are released to start a new cycle. Control submerged cultures, after 20 hours incubation, were masively sporulated (Fig. 1A), but in the presence of L-ethionine, growth was slightly enhanced, and substrate mycelium development was notably stimulated; morphological indications of differentiation were absent (Fig. 1C). In the presence of the nucleosides, however, reproductive hyphae were formed (Fig. 1B). Under the described conditions, inhibition of sporulation continued beyond 7 days of incubation. 5-AC inhibited spore germination in submerged cultures (and also growth) when added during early logarithmic phase. Concentrations higher than 1 mM significantly inhibited growth at any time, as well as 5-dAC concentrations over 75 μM. Cordycepin inhibited sporulation only when growth was completely inhibited, while lower concentrations that allowed growth had no effect on sporulation (see below). Adenosine slightly stimulated growth and delayed sporulation in GYM and had no effect in SPM media where S.antibioticus differentiation is repressed at different stages[27]. Interestingly, SAM showed an enhancing effect on sporulation in SM.

TABLE 3

INHIBITOR	CONCENTRATION	GROWTH	SPORULATION
Control		0.41-0.44	+
5-azacytidine	0.5 mM	0.40-0.42	-
5-fluoro-2'-deoxycytidine	2 mM	0.43-0.45	+
L-ethionine	1.5 mM	0.53-0.57	-
Cycloleucine	2 mM	0.45-0.46	+
Dimethylsulfoxide	500 µM	0.38-0.40	+
Cordycepin	2 mM	0.005(NG)	-
Adenine	1-5 mM	0.44-0.45	+
L-methionine	1 mM	0.40-0.45	+
Adenosine	1-5 mM	0.59-0.68	+
S-adenosyl-L-methionine	1 mM	0.43-0.44	++

Effect of DNA-methylation inhibitors on Streptomyces antibioticus ETHZ 7451 submerged sporulating cultures[26] (see Table 1). Growth is given as the range of DO_{600} of different replicas. NG: No Growth.

Reversion of 5-AC effect by pyrimidine nucleosides. Micromonospora chalcea growth in submerged cultures is inhibited by 5-AC with higher efficiency than in surface cultures, as observed for Streptomyces cultures, being affected by concentrations higher than 15 µM. On the other hand, at subinhibitory concentrations, a stimulation of vegetative mycelium was noted. To test whether 5-AC exerted its effect by blocking the "de novo" synthesis of pyrimidine nucleosides, as already described in Bacillus[31], 0.2 mM pyrimidine nucleosides (cytidine, uridine, thymidine) were added to cultures with 0.1 mM 5-AC. Only cytidine could partially revert the 5-AC effect.

Effect of nucleic acids and protein inhibitors on S.antibioticus submerged sporulation. To test the effect of macromolecular synthesis inhibition on sporulation, several

inhibitors of DNA, RNA and protein biosynthesis (Table 1) were added to submerged cultures of S. antibioticus ETHZ 7451. In all cases, only under conditions of total absence of growth, was sporulation blocked. Subinhibitory concentrations of any of the antibiotics allowed sporulation to occur. Thus, partial growth inhibition, seems not to be the primary cause of sporulation inhibition. This was observed with DMSO, wich affects growth, but not sporulation.

DISCUSSION

We have shown that some DNA-methylase inhibitors prevent Streptomyces and Micromonospora differentiation under a variety of physiological conditions. None of the studied compounds is totally specific, and unknown side effects may occur, but some possibilities can be discarded. 5-AC, the most specific (after 5-dAC), inhibits protein biosynthesis in E. coli[32], but not in S. antibioticus under these conditions (I.S. Novella, unpublished results). Pyrimidine nucleoside "de novo" synthesis is inhibited by 5-AC in Bacillus subtilis[31], causing interference with growth. However, at least for Micromonospora chalcea, and probably for the other actinomycetes, this was not the mechanism of action, as only cytidine was able to partially revert its effect. The 5-AC concentration that inhibited Micromonospora sporulation (about 2.5 μM) was similar to that needed for sporulation impairment in Bacillus[16], but much lower than that used to block growth in E. coli (about 0.1 mM) and Streptomyces differentiation (0.5-1 mM), or to induce cell culture differentiation in higher organisms[33]. It must be noted that sinefungin causes sporulation inhibition in a similar maner, and inhibits nucleic acid, but not protein, methyltransferases[23]. It is also important to note that Streptomyces DNA methyltransferases of cultures grown in the presence of both 0.5 mM 5-AC and 1.5 mM L-ethionine have lower activity than corresponding untreated controls[26] (I.S. Novella and J. Sánchez, unpublished results). Sensitivity variations between Micromonospora and Streptomyces, and also

among strains of these genera, could be attributed to diferential permeability caused by cell wall and coat composition. The absence of effects of cycloleucine and 5-fluoro-2'-deoxycytidine could also have this explanation, though another possibility for lack of effect of 5-fluoro-2'-deoxycytidine is a rapid deamination that it can undergo in the cytoplasm[28]. Submerged sporulation as seen in S. antibioticus ETHZ 7451 seems to be more sensitive to inhibitors than surface sporulation. This phenomenon had been previously described in this and another S. antibioticus strain with the nutritional repressive conditions which impair sporulation in both environments[34]. The reversion observed on 5-AC treated solid cultures could, in particular, be caused by analogue instability or, more probably, by its exhaustion around colonies. As previously reported[33], the effect caused by 5-AC was achieved with lower concentration of 5-dAC. L-ethionine blocks Streptomyces differentiation in an earlier developmental stage than 5-AC. This could be due to side effects caused by this compound, including its incorporation into proteins[4]. However, severe alterations in metabolic functions are not likely to occur in Streptomyces, as the analog does not impair growth. Our results suggest that DNA hypomethylation is characteristic of vegetative growth while reproductive stages require some extent of methylation. Differential results obtained with 5-AC and L-ethionine are not surprising because of the less specific nature of L-ethionine inhibition. As mentioned above, in addition to DNA methylation, other biochemical routes in which SAM is used as methyl-donor are likely to be affected, such as phospholipid formation[14]. Partial growth inhibition can not explain sporulation impairment, because protein, DNA and RNA synthesis must be completely blocked to prevent sporulation, and even lack of growth under starvation conditions is not enough to impair differentiation[26]. Results of sporulation enhancenment by SAM are similar to those reported in Bacillus[14,17]; SAM (the methyl donor) could stimulate DNA-methylation. Adenine and L-methionine are

probably used for macromolecular biosynthesis of nucleic acids and proteins respectively; adenosine is clearly used as a nitrogen source, and the delaying effect on sporulation could be related to the stringent response and changes in of ppGpp and GTP pools[35,36].

These results suggest that Streptomyces and Micromonospora differentation could be controlled by DNA methylation. This hypothesis is supported by the fact that methylated base composition (5-methylcytosine and N^6-methyladenine) varies during the life cycle[12]. However inhibition of sporulation by the tested compounds can not be inequivocally asigned to DNA-methylase inhibition. To clarify this our laboratory is being currently investigating their effect on protein, DNA and RNA methylases.

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