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DNA from *Aspergillus flavus* contains 5-methylcytosine

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Abstract

DNA from *Aspergillus* sp. has been reported not to contain 5-methylcytosine. However, it has been found that *Aspergillus nidulans* responds to 5-azacytidine, a drug that is a strong inhibitor of DNA methyltransferases. Therefore, we have re-examined the occurrence of 5-methylcytosine in DNA from *Aspergillus flavus* by using a highly sensitive and specific method for detection of modified bases in genomic DNA comprising high-performance liquid chromatography separation of nucleosides, labeling of the nucleoside with deoxynucleoside kinase and two-dimensional thin-layer chromatography. Our results show that 5-methylcytosine is present in DNA from *A. flavus*. We estimate the relative amounts of 5-methylcytosine to cytosine to be approximately 1/400. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: 5-Methylcytosine; DNA methylation; Fluffy phenotype; Fungal development; *Aspergillus flavus*

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1. Introduction

Aspergillus species are of major importance to industry [1]. They are used for food fermentation and as a source of highly active, stable enzymes [2]. Nevertheless, some species, such as *Aspergillus flavus* and *Aspergillus parasiticus*, are a threat to commercially important crops such as corn, cotton, ground and tree nuts [3]. They are also a threat to human health, since they produce highly toxic and carcinogenic aflatoxins [4] which are among the major risk factors for liver cancer [5]. The annual economic burden from aflatoxin contamination and prevention has been estimated to be greater than $100 million [6]. Furthermore, *Aspergillus* infection can cause severe pulmonary disease in immunocompromised patients [7].

Aspergillus sp. are filamentous fungi which have no obvious sexual stage. Prolonged propagation without sporogenesis results in strain degeneration involving chromosomal instability, loss of or reduction in the ability to form spores, and loss of the ability to synthesize aflatoxin or its precursors [8,9]. Colonies displaying strain degeneration have abundant aerial mycelia and so are designated as having a 'fluffy' phenotype [8]. This phenotype has also been observed in transformant colonies in which genes involved in early regulation of development in *Aspergillus nidulans* were mutated [10].

Other filamentous fungi like *Ascobulus immersus* or *Neurospora crassa* are known to contain 5-methylcytosine at levels of methylcytosine/cytosine of about 1.5/100 (*N. crassa*: [11]). In *N. crassa* and *A. immersus*, DNA methylation is involved in premeiotic silencing of duplicated genes, either by methylation induced premeiotically (MIP) in *Ascobulus*, or by repeat-induced point mutation (RIP) in *Neurospora* [12]. Usually RIP is accompanied by de novo methylation, although methylation is not essential for RIP to occur. During RIP and MIP, the methylation occurs at cytosine residues in a variety of sequences and is not mainly found at CpG or CpNpG sequences as it is for animals and vascular plants. *A. immersus* contains two active DNA methyltransferases, Masc1 which is involved in MIP [13], and Masc2 whose biological function is not known [14,15]. In *N. crassa* only one Mtase, Dim-2, has been identified [16]. In contrast to these species, no cytosine methylation was detected in *Aspergillus* by TLC analysis, cleavage with CpG methylation-sensitive restriction endonucleases, and nearest neighbor analysis [17,18].

Surprisingly, it has also been observed that treatment of

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A. nidulans with 5-azacytidine induces a fluffy phenotype. This change has been linked to a specific genetic locus, *fluF*, that has yet to be characterized [17,19]. Since 5-azacytidine is known to be a very potent DNA methyltransferase inhibitor, this observation is not easily interpreted in the light of the apparent absence of DNA methylation in *Aspergillus*. However, important methodological improvements have been developed recently that allow detection of very low levels of DNA cytosine methylation [20]. For example, it could be shown only last year that *Drosophila melanogaster*, a species that was believed to be devoid of DNA methylation, indeed contains 5-methylcytosine [20,21]. This finding prompted us to re-investigate the occurrence of 5-methylcytosine in *A. flavus* using a highly sensitive method that combines high-performance liquid chromatography (HPLC) separation of nucleosides, radioactive labeling of the nucleosides by deoxynucleoside kinase (dNK) and two-dimensional thin-layer chromatography (2D-TLC).

2. Materials and methods

2.1. *Aspergillus strains and growth conditions*

In this study *A. flavus* strain 13 (American Type Culture Collection 96044) [22] was used. The DNA was prepared from 1- and 3-day-old cultures grown from a spore suspension on the chemically defined medium of Adye and Mateles [23] with shaking at 30°C.

2.2. *DNA preparation*

DNA was prepared by the method of Chang et al. [24]. The $A^{260\ \mathrm{nm}}/A^{280\ \mathrm{nm}}$ was between 1.75 and 1.8 in all preparations. After purification, the DNA was treated with a mixture of RNaseA and RNaseT1 (RNase PLUS, 5 Prime, 3 Prime, Inc., Paoli, PA, USA), proteinase K (Sigma, St. Louis, MO, USA), and extracted two times with phenol/chloroform. Aliquots of the preparations were fully digested by 50 U *Eco*RI at a concentration of 1 μg/40 μl.

2.3. *HPLC analysis*

HPLC separation of the nucleosides was carried out basically as described [25]. About 2 μg DNA of each preparation was degraded to nucleosides using P1 nuclease, *Serratia marcescens* nuclease and Shrimp alkaline phosphatase (Amersham) and subjected to reversed phase HPLC. The following gradient was used at a flow rate of 0.5 ml min^{-1}: $t=0$ min: 100% solvent A (100 mM triethylammonium acetate, pH 6.5); linear gradient to $t=15$ min: 15% solvent B (100 mM triethylammonium acetate, pH 6.5, 30% acetonitrile); linear gradient to $t=40$ min: 100% solvent B; $t=50$ min: 100% solvent B. As references a mixture of all four deoxynucleotides (Sigma) and two oligonucleotides (oligo-C: d(AGA CGG TGG TCG GGT TCG ACG) and oligo-^{m}C: d(GAA G^{m}CT GGG A^{m}CT T^{m}C^{m}C GGG AGG AGA GTG ^{m}CAA)) were hydrolyzed to nucleosides following the same protocol and subjected to HPLC separation. Under these conditions, cytosine elutes after 11 ± 0.5 min and methylcytosine after 13.5 ± 0.5 min. Fractions were collected for 1-min intervals during the entire run. The fractions were dried in vacuum, washed twice in water and finally dissolved in 15 μl water.

2.4. *dNK labeling*

Labeling of the deoxynucleosides in the HPLC fractions was carried out using the multi-substrate deoxyribonucleoside kinase from *D. melanogaster* essentially as previously described [20]. The HPLC fractions were dissolved in 15 μl water, 2 μl of the dissolved samples was incubated with 30 nM dNK in 15 μl buffer (50 mM Tris–HCl pH 8.5, 5 mM $MgCl_2$) containing 0.25 μl [γ-^{32}P]ATP (370 MBq ml^{-1}, NEN) for 3 h at 37°C. Prior to the phosphorylation reaction, the deoxyribonucleotide kinase was incubated with 40 nM unlabeled ATP for 10 min to minimize the signals obtained from endogenously bound deoxynucleosides.

2.5. *2D-TLC analysis*

2D-TLC analyses were carried out as described [20]. 2 μl of the radioactive sample was applied to cellulose TLC plates (Cellulose CEL 400-10, 20×20 cm, Macherey-Nagel, Düren, Germany) at ambient temperature. The first dimension was run in isobutyric acid/water/ammonia (66:33:1, v/v/v). The plate was dried in air and developed

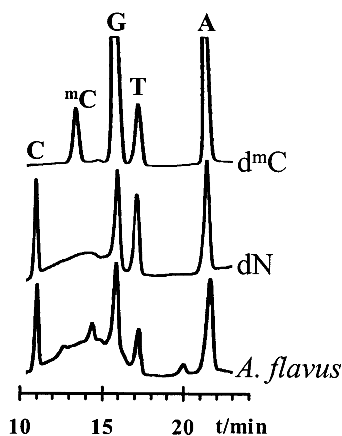

Fig. 1. HPLC analysis of DNA from *A. flavus*. DNA was digested to nucleosides and subjected to HPLC analysis. The figure shows $A^{260\ \mathrm{nm}}$ traces obtained in the HPLC experiments with a reference deoxynucleoside mixture (dN), with a degraded oligonucleotide that contains 5-methylcytosine, guanine, thymine and adenine (d^{m}C) and with DNA from *A. flavus* isolated from 3-day-old cultures. The elution positions of deoxycytidine (C), 5-methyldeoxycytosine (^{m}C), deoxyguanosine (G), thymidine (T) and deoxyadenosine (A) are indicated.

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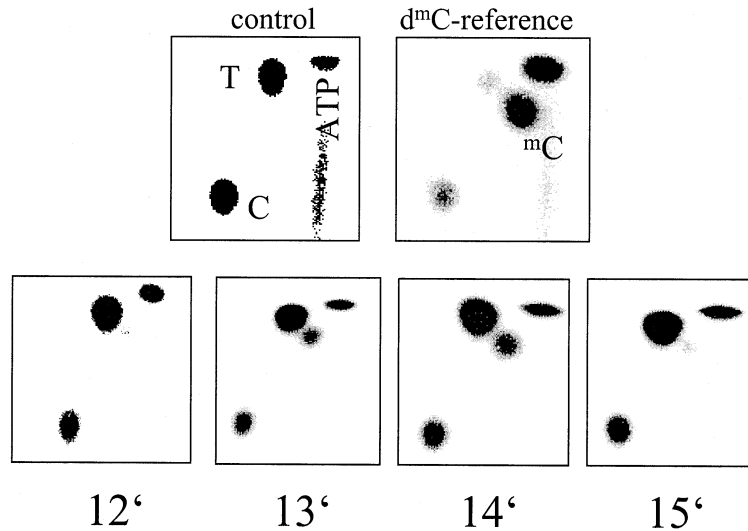

Fig. 2. Detection of 5-methylcytosine in the DNA from *A. flavus*. The upper row shows two reference plates. Control: The dNK was incubated only with buffer and [γ-^{32}P]ATP and the reaction mixture analyzed by 2D-TLC. The thymidine and deoxycytidine peaks are due to deoxynucleosides bound to the enzyme. dmC-reference: 2 μl of the 5-methylcytosine fraction of oligo-^{m}C were labeled with dNK and subjected to 2D-TLC. In the lower row 2 μl of different HPLC fractions of the *A. flavus* DNA were incubated with dNK and analyzed by 2D-TLC.

in the second dimension with isopropanol/concentrated HCl/water (70:15:15, v/v/v). The plate was dried and the radioactivity analyzed using an instant imager (Canberra Packard).

3. Results

Reversed phase HPLC allows reproducible separation of all four normal nucleosides and modified ones like 6-methyladenosine, 4-methylcytidine and 5-methylcytidine [20,25,26]. The combination of HPLC with dNK labeling and 2D-TLC provides high specificity and sensitivity for the detection of methylcytosine in genomic DNA as demonstrated by the ability of the procedure to detect 5-methylcytosine in DNA from *D. melanogaster*, which previously was believed to be devoid of modified bases [20]. Here we aimed to investigate if 5-methylcytosine is present in the DNA from *A. flavus*, a filamentous fungus involved in aflatoxin biosynthesis that has no known sexual cycle. The DNA from *A. flavus* was prepared from cultures 1 and 3 days of age. 2 μg of the DNA was enzymatically hydrolyzed to nucleosides and applied to reversed phase HPLC. The elution positions of deoxycytidine, deoxyguanosine, thymidine, and deoxyadenosine were determined using a set of standard nucleotides and an oligonucleotide (oligo-^{m}C) that contains 5-methyldeoxycytosine but no cytosine (Fig. 1). As shown in Fig. 1, a clear separation of 5-methylcytosine from deoxycytosine and deoxyguanosine is obtained. In addition, a second oligonucleotide (oligo-C) that does not contain modified bases was used to confirm the elution positions of the unmodified deoxynucleosides (data not shown). The $A^{260\,nm}$ trace of the HPLC runs of both DNA samples from *A. flavus* looked virtually identical. In either case no peak was visible at the position of 5-methylcytosine[2] (Fig. 1) indicating that the ratio of 5-methylcytosine and cytosine cannot be higher than 1/50.

The eluate from the HPLC was collected at 1-min intervals. Deoxynucleosides were radioactively labeled with dNK and the 5′-deoxynucleotides analyzed by 2D-TLC. If dNK is incubated only with water, deoxycytidine and thymidine are always observed, demonstrating that the enzyme carries endogenously bound cytidine and thymidine (Fig. 2A) which we always found co-purified with the enzyme. To determine the position of 5-methylcytosine on the 2D-TLC plate, an aliquot of an HPLC fraction containing 5-methylcytosine (12–14-min fractions of oligo-^{m}C) was labeled with dNK (Fig. 2B). We then incubated aliquots of the fractions obtained after HPLC separation of DNA from *A. flavus* with dNK and [γ-^{32}P]ATP. As shown in Fig. 2C, 5-methylcytosine spots are observed in the 13-min and 14-min fractions which is in perfect agreement with the elution profile of 5-methylcytosine from the HPLC column (cf. Fig. 1). No methylcytosine is observed in the corresponding HPLC fractions of the deoxynucleotide standard and the oligonucleotide that does not contain modified cytosine (data not shown). These results clearly indicate that DNA from *A. flavus* contains 5-methylcytosine.

To estimate the amount of 5-methylcytosine in relation to cytosine, we pooled both methylcytosine and both cytosine fractions from the *Aspergillus* DNA HPLC run and added the same volumes of the methylcytosine sample and

[2] The peaks eluting at 12.5 and 14.5 min in the *A. flavus* chromatogram are not due to nucleosides, because no additional spots are observed in the 2D-TLC analysis (see Fig. 2). Therefore, we think these peaks either arose from incompletely digested di-, tri- and oligodeoxynucleotides or from RNA contaminations. We tried different conditions for the nucleolytic digestion, but were not able to remove these peaks.

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the cytosine sample in different dilutions to the labeling mixture. Addition of one aliquot of the cytosine sample diluted 1/10 000 did not alter the amount of methylcytosine detected. If the cytosine sample was used in a dilution of 1/1000, the methylcytosine signal was reduced by 30%. Addition of the cytosine sample in a 1/100 dilution reduced the methylcytosine spot to ≤10%. On the basis of these results, the ratio of cytosine and 5-methylcytosine in DNA from *A. flavus* can be estimated to be approximately 400/1.

4. Discussion

In eukaryotes, methylation of DNA usually leads to repression of gene expression (reviews: [27–30]). It serves as a device for epigenetic gene control that is employed for various different functions like control of development, protection of the genome from foreign DNA, X-chromosome inactivation, and parental imprinting. Therefore, presence of 5-methylcytosine in an organism has many important consequences for its biology and its possibilities for gene regulation. We show here that DNA from *A. flavus*, like that of other filamentous fungi (*N. crassa*, *A. immersus*), contains modified cytosines. This result is in agreement to the recent discovery of a DNA methyltransferase homologous to the *A. immersus* Masc1 enzyme in *A. nidulans* (D. Lee, M. Freitag, E. Selker and R. Aramayo, personal communication).

At the present state of knowledge, we can only speculate on the possible biological function of DNA methylation in *Aspergillus*. In *A. flavus* and *A. parasiticus* integration of a duplicated gene at a site distant from the wild-type copy resulted in a 500-fold decrease in transcriptional activity [31]. It is not known if methylation could play a role in this gene silencing process. In *A. parasiticus* the whole *aflR* gene, which encodes the aflatoxin pathway-specific transcription factor, AflR, and its promotor, is duplicated. Although the duplicated copy has only a few mutations which did not affect protein or promotor function, strains in which the primary copy was disrupted showed no transcriptional activity (J.W. Cary, unpublished work). This phenomenon resembles post-transcriptional gene silencing, that might (but need not) include DNA methylation [32]. A process similar to MIP in *Ascobolus* might suppress homologous recombination between dispersed DNA repeats and thereby preserve genome integrity [33]. Another possible role for cytosine methylation is to inhibit transposon movement in a similar manner to that discovered in higher plants [34]. Transposable elements have been identified in filamentous fungi [35] including some *Aspergillus* species [36–38]. Evidence has been presented for methylation-associated silencing of an exogenously introduced transposon in *Neurospora* [39]. Finally, the early experiments by Tamame et al. [17], in which developmental abnormalities were induced by the DNA methyltransferase inhibitor 5-azacytidine, point towards a role for DNA methylation in *Aspergillus* development that might be similar to its role in *A. immersus*, where a Masc1 knock-out mutant was no longer able to undergo sexual reproduction [13].

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