Cytosine DNA Methylation Is Found in Drosophila melanogaster but Absent in Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Other Yeast Species

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ABSTRACT: The methylation of cytosine to 5-methylcytosine (5-meC) is an important epigenetic DNA modification in many bacteria, plants, and mammals, but its relevance for important model organisms, including Caenorhabditis elegans and Drosophila melanogaster, is still equivocal. By reporting the presence of 5-meC in a broad variety of wild, laboratory, and industrial yeasts, a recent study also challenged the dogma about the absence of DNA methylation in yeast species. We would like to bring to attention that the protocol used for gas chromatography/mass spectrometry involved hydrolysis of the DNA preparations. As this process separates cytosine and 5-meC from the sugar phosphate backbone, this method is unable to distinguish DNA- from RNA-derived 5-meC. We employed an alternative LC−MS/MS protocol where by targeting 5-methyldeoxycytidine moieties after enzymatic digestion, only 5-meC specifically derived from DNA is quantified. This technique unambiguously identified cytosine DNA methylation in Arabidopsis thaliana (14.0% of cytosines methylated), Mus musculus (7.6%), and Escherichia coli (2.3%). Despite achieving a detection limit at 250 attomoles (corresponding to <0.00002 methylated cytosines per nonmethylated cytosine), we could not confirm any cytosine DNA methylation in laboratory and industrial strains of Saccharomyces cerevisiae, Schizosaccharomyces pombe, Saccharomyces boulardii, Saccharomyces paradoxus, or Pichia pastoris. The protocol however unequivocally confirmed DNA methylation in adult Drosophila melanogaster at a value (0.034%) that is up to 2 orders of magnitude below the detection limit of bisulphite sequencing. Thus, 5-meC is a rare DNA modification in drosophila but absent in yeast.

A covalently modified DNA base, 5-methylcytosine (5-meC), is widely found in bacteria, plants, and mammalian cells and is associated with the epigenetic regulation of gene expression. However, this DNA modification is not ubiquitous. 5-meC is thought to be absent in the DNA of many species, including popular laboratory model organisms such as Caenorhabditis elegans. Other organisms might possess low amounts of cytosine DNA methylation, including Neurospora crassa, Dictyostelium discoideum, Schistosoma mansoni, and Drosophila melanogaster, for some of which DNA methylation was initially thought to be absent. In several of these cases, the content of DNA methylation is at the edge of the detection limit of bisulphite sequencing approaches, currently the dominating technique for analysis of DNA methylation. Despite that bisulphite sequencing gives highly valuable information about the sequence context of methylated cytosine, it has limitations at the lower concentration range: incomplete bisulphite conversion of unmethylated cytosines and misalignment of sequencing reads in the mapping process in repetitive, telomeric, and GC-rich regions result in false positive rates of ~0.5% and higher. For this reason, the existence and biological function of low methylation levels is still ambiguous and/or debated in some species.

Until recently, also DNA of budding yeast Saccharomyces cerevisiae has been considered to be free of 5-methylcytosine. Despite this modification had been described in the first studies that date back to the late 1970’s, later studies did not confirm DNA methylation of S. cerevisiae. Yeast DNA is not cut by methylation-dependent restriction endonucleases, and 5-meC was not found in DNA digests analyzed by HPLC/UV−VIS
where a detection limit of 1 per 3100 to 6000 residues was achieved. Recently however, Tang et al. challenged this result. With the use of a GC–MS method, a much lower detection limit (6.4 fmol) compared to the HPLC protocols was
achieved. This protocol detected 5-meC in DNA extracts of three budding yeast laboratory strains.\(^6\) Moreover, this study reported DNA methylation in several other yeast species as well, which included the medically important tropical yeast \textit{Sacharomycodes boulardi} and the popular laboratory model fission yeast (\textit{Schizosaccharomyces pombe}). Indeed, the evidence for DNA methylation is debated in the latter species. In difference to \textit{S. cerevisiae}, \textit{S. pombe} possesses a cytosine methyltransferase homologue of the DNMT2 family, termed Pombe MethylTransferase 1 (Pmt1).\(^1^4\) Pmt1, however, appears to be specific for tRNA modification.\(^1^5\) The 5-meC contents reported by Tang et al. ranged from 0.036% for \textit{S. boulardi} to 0.128% in the common \textit{S. pombe}.\(^1^6\) These conclusions are challenged by the application of an improved technique. Employing a liquid chromatography selective reaction monitoring (LC–SRM) method that is insensitive to contamination with RNA-derived methylcytosine, we achieve a detection limit of 250 attomoles for methylcytosine (around 25 times more sensitive compared to the previous study).\(^1^3\) We detect no cytosine DNA methylation in the different yeast species \textit{S. cerevisiae}, \textit{S. boulardi}, \textit{S. paradoxus}, \textit{S. pombe}, and \textit{P. pastoris}. In contrast, the protocol unambiguously detected and accurately quantified DNA methylation in \textit{Mus musculus}, \textit{Arabidopsis thaliana}, \textit{Escherichia coli}, and at a low but significant level in \textit{Drosophila melanogaster}.\(^1^6\)

\section*{RESULTS AND DISCUSSION}

Table 1. Content of 5-Methyldeoxycytidine (5-meC) in DNA Preparations of Ten Species and the Estimated Average Numbers of 5mdC per (Haploid) Genome of \textit{A. thaliana}, \textit{M. musculus}, \textit{D. melanogaster}, \textit{E. coli}, \textit{S. cerevisiae}, \textit{S. boulardi}, \textit{S. paradoxus}, \textit{P. pastoris}, and \textit{S. pombe}\(^a\)

| species                 | genome size MB | % methylation | Est. no. 5mdC per MB | Est. no. 5mdC/genome |
|--------------------------|----------------|---------------|----------------------|----------------------|
| \textit{A. thaliana}     | 119.7          | 14 ± 1.5      | \(5.0 \times 10^7\)  | \(6.0 \times 10^8\)  |
| \textit{M. musculus}     | 2800           | 7.6 ± 0.8     | \(3.2 \times 10^4\)  | \(8.9 \times 10^7\)  |
| \textit{D. melanogaster} | 139.5          | 0.034 ± 0.013 | \(1.4 \times 10^2\)  | \(2 \times 10^4\)    |
| \textit{E. coli DH5a}    | 4.6            | 2.3 ± 0.1     | \(1.2 \times 10^4\)  | \(5.4 \times 10^4\)  |
| \textit{E. coli GM2929 (dcm-6)} | 4.6            | 0.016 ± 0.002 | 81                   | 3.8 \times 10^2      |
| \textit{S. cerevisiae D27–310B**} | 12.5           | not detected (<0.002) | not detected (LOD = 8) | not detected (LOD = 96) |
| \textit{S. cerevisiae AWRI 796**} | 12.5 (b.f.) | not detected | not detected | not detected |
| \textit{S. cerevisiae SCD0308} | 12.5 (b.f.) | not detected | not detected | not detected |
| \textit{S. cerevisiae SaflagerW-34/70} | 12.5 (b.f.) | not detected | not detected | not detected |
| \textit{S. boulardi CBS 5926} | 11.4          | not detected  | not detected | not detected |
| \textit{S. paradoxus KPN3829} | 11.8          | not detected  | not detected | not detected |
| \textit{S. pombe 972s}  | 13.8           | not detected  | not detected | not detected |
| \textit{P. pastoris SMD1168} | 9.4           | not detected  | not detected | not detected |

\(^a\)Total 5mdC per genome and MB average estimations were calculated based on genome size and GC content.\(^2^9–35\) b.f. = bona fide. Est. = estimated.

\footnotesize{\textsuperscript{17} we obtained 5-meC concentration values for \textit{S. cerevisiae} DNA fully comparable to those reported by Tang et al. (data not shown), confirming the precision of the GC–MS technology. Surprisingly, however, this method revealed significant 5-meC content also in negative control DNA purified from the \textit{E. coli} strain GM2929 (Marinus, CGSC no: 7080). This was unexpected, as this \textit{E. coli} strain is deficient in bacterial DNA methyltransferases, including the \textit{dcm-6} allele and is used in laboratories around the world to produce nonmethylated DNA for cloning experiments.\(^1^9,2^0\)} In the search for a potential source of the methylated cytosine in GM2929 DNA preparations, we considered copurified methylcytosine derived from RNA as its potential source. Cytosine methylation has been found as an abundant modification on different RNA species, especially on iRNA in prokaryotes and tRNA in eukaryotes.\(^2^1–2^3\) In order to setup a quantification method that restricts the detection of 5-meC to DNA-derived nucleotides, we used a protocol based on enzymatic release of nucleosides using the enzyme DNA Degradase Plus (ZymoResearch).\(^2^4\) The enzymatic digest preserves the base coupled to the deoxyribose (nucleoside, Figure 1a). This enables the separation of the DNA-derived deoxycytidine and the RNA-derived (hydroxyl)-cytidine by mass spectrometry due to significant mass difference. Samples processed in this manner were analyzed using a state of the art HPLC (Agilent 1290 Infinity) coupled to a triple quadrupole mass spectrometer (Agilent 6460), set to quantify deoxyribosecytosine (Figure 1b, upper panel) and deoxyribose-5-methylcytosine (5-methylcytidine, Figure 1b, middle panel).

This DNA specific method unambiguously detected deoxycytidine and 5-methyldeoxycytidine in purified standards over a concentration range of 3 orders of magnitude. 5-Methyldeoxycytidine and deoxycytidine were quantified by external calibration within the dynamic range of the analytical method (\(R^2 = 0.9999\) and \(R^2 = 0.9965\), respectively, Figure 1b). A limit of detection at 250 attomol per injection for 5-meC was obtained (Figure 1b, left inset). The absence of an inferring matrix effect of the DNA digest sample was confirmed by reproducing the limit of detection and linear range by standard addition of 5-meC to a fully processed sample of \textit{S. cerevisiae} DNA (Figure 1b, right inset). This method clearly detected and chromography-tandem mass spectrometry (LC–MS/MS),\(^1^7\) we obtained 5-meC concentration values for \textit{S. cerevisiae} DNA fully comparable to those reported by Tang et al. (data not shown), confirming the precision of the GC–MS technology. Surprisingly, however, this method revealed significant 5-meC content also in negative control DNA purified from the \textit{E. coli} strain GM2929 (Marinus, CGSC no: 7080). This was unexpected, as this \textit{E. coli} strain is deficient in bacterial DNA methyltransferases, including the \textit{dcm-6} allele and is used in laboratories around the world to produce nonmethylated DNA for cloning experiments.\(^1^9,2^0\) In the search for a potential source of the methylated cytosine in GM2929 DNA preparations, we considered copurified methylcytosine derived from RNA as its potential source. Cytosine methylation has been found as an abundant modification on different RNA species, especially on iRNA in prokaryotes and tRNA in eukaryotes.\(^2^1–2^3\) In order to setup a quantification method that restricts the detection of 5-meC to DNA-derived nucleotides, we used a protocol based on enzymatic release of nucleosides using the enzyme DNA Degradase Plus (ZymoResearch).\(^2^4\) The enzymatic digest preserves the base coupled to the deoxyribose (nucleoside, Figure 1a). This enables the separation of the DNA-derived deoxycytidine and the RNA derived (hydroxyl)-cytidine by mass spectrometry due to significant mass difference. Samples processed in this manner were analyzed using a state of the art HPLC (Agilent 1290 Infinity) coupled to a triple quadrupole mass spectrometer (Agilent 6460), set to quantify deoxyribosecytosine (Figure 1b, upper panel) and deoxyribose-5-methylcytosine (5-methylcytidine, Figure 1b, middle panel).
quantified S-meC in DNA samples of *A. thaliana* leaves (14.0% of deoxycytidines methylated), mouse liver (7.6%), and methylation competent *E. coli* K12 cells (strain DH5αpha 2.3%), confirming DNA methylation as expected (Figure 1, panels b and c). Taking into account the genome size and GC content, these values allowed an estimation about the number of methylated cytosine residues per genome (*A. thaliana*, 6 × 10^8; *M. musculus*, 9 × 10^9; *E. coli*, 5 × 10^8) (Table 1). Moreover, this protocol confirmed the expected decline in methylation in the DNA methyltransferases deficient (*dem-6*) K12 derivate GM2929 (Figure 1b, Table 1). Compared to the methylation competent K12 strain DH5α, the content of methylated cytosines was reduced to 0.016% (equaling <500 modified cytosine bases per genome), which corresponds to a reduction in DNA methylation of 99.3%.

Whereas, unmethylated cytidines were readily detected in all yeast species (Figure 1b, upper panel), the protocol applied on *S. cerevisiae*, *S. boulardii*, *S. paradoxus*, *P. pastoris*, and *S. pombe* DNA did not detect any evidence for the content of 5-methyldeoxycytidine (Figure 1b, lower panel; Table 1). Taking into account the limit of detection at 250 attomol for methyldeoxycytidine, its content in yeast would be lower than 0.00002 per deoxycytidine (Table 1). Considering the importance of budding yeast in basic research and industry, the analysis was conducted on five different *S. cerevisiae* yeasts. These included the laboratory strains BY4741 (in its prototrophic version, BY4741-pHLUM) androgenomlym, which had yielded the highest *S. cerevisiae* S-meC content in the study by Tang et al., and the strain D273–10B that was used in the seminal study that claimed for the first time the absence of DNA methylation in *S. cerevisiae*. The measurements were conducted on DNA purified both from cells grown to stationary and exponential phase. Under both conditions DNA methylation remained undetectable. In addition, the absence of methylation was confirmed in industrial yeast. Analysis of the vine production strain AWRI 796, the lager/pilsner beer production yeast SCHA000308 (Ruf, Germany) revealed no evidence for DNA methylation either (Table 1).

The results obtained in budding yeast were representative for other yeast species. We measured DNA samples obtained from *S. boulardii*, a tropical yeast of medicinal use isolated from lychee and mangosteen fruit in 1923, *S. paradoxus*, a yeast species living on the bark of deciduous trees, the common laboratory models fission yeast *S. pombe*, and the methylo-trophic yeast commonly used in protein production, *P. pastoris*. DNA methylation was not detected in any of these yeast species either (Table 1).

Finally, we applied this protocol to DNA obtained from *D. melanogaster* DNA. The fruit fly is a common model organism, but the role and existence of its DNA methylation is the subject of an ongoing debate. We could clearly and unambiguously detect *Drosophila* cytosine DNA methylation (Figure 1b). Revealing 0.034% of cytosines methylated, the total number of methylecytosine in the Drosophila genome is thus estimated to be in the range of 2 × 10^8 modified bases per genome (Table 1). This result thus confirms methylation in drosophila, reveals however a lower content as compared to estimations obtained with previous methods. The total content of methylated cytosines in *Drosophila* is thus comparable to those in the *E. coli* genome, albeit the latter genome is substantially smaller (Table 1). This value is several orders of magnitude above the detection limit of the LC–MS/MS method (Figure 1). It is however 10–100 fold below the error rate/detection limit of bisulphite sequencing. Bisulphite sequencing, at least in its current implementation, is thus not applicable for the analysis of *Drosophila* DNA methylation.

**CONCLUSIONS**

An LC-SRM method specific to DNA-derived nucleosides was used to assess cytosine DNA methylation in different model organisms. A detection limit of 250 attomol for methyl-deoxycytidine, which corresponded to one modified base per 50000 deoxycytidines, was achieved. As expected, cytosine DNA methylation was detected and could be precisely quantified in *A. thaliana*, *M. musculus*, and *E. coli* DNA. Substantially lower, but unequivocal, evidence for DNA methylation was obtained in *D. melanogaster* DNA. However, DNA methylation was not detected in laboratory and industrial strains of *S. cerevisiae*, *S. pombe*, *S. boulardii*, *S. paradoxus*, and *P. pastoris*. In summary, these results reveal that methylcytosine quantification methods that are based on hydrolytic DNA cleavage are sensitive to copurification artifacts. Moreover, species such as *Drosophila* contain quantities of DNA methylation too low to be measured by bisulphite sequencing. Importantly however, this study demonstrates that while a low amount of DNA methylation is detectable in *D. melanogaster*, yeast species do not possess cytosine DNA methylation.

**MATERIALS AND METHODS**

**Chemical and Reagents.** Chemical standards were obtained from Sigma at a purity >99% ([2′-deoxycytidine (dC), SD3897], cytidine (C, C122106), 5-methylcytidine (5mC, M4254]) and the 5-methyl-2′-deoxycytidine (5mDdC, sc-278526, purity >99%) from Santa Cruz Biotechnology. UPLC-grade methanol, water, and formic acid were purchased from Sigma-Aldrich. RNase A was purchased from Roche (10109169001).

**Yeast and Bacteria Culture Conditions.** The *S. cerevisiae* strains BY4741 pHLUM, D273–10B, and AWRI 796 (NCBI Taxon ID: 764097), as well as industrial yeast SCHA000308 and Slaflager W-34/70, *S. boulardii* CBS 5926, *S. paradoxus* KPN3829, and *P. pastoris* SMD1168 were inoculated in 300 mL YPD [yeast extract (10 g/L), peptone (20 g/L), and 2% glucose] with 0.2 OD_{600} of yeast culture and incubated at 30 °C, 200 rpm. *S. pombe* strain 972h- was inoculated in 300 mL YED [yeast extract (5 g/L), 3% glucose]. Yeast cells were collected both at exponential (OD_{600} = 2) and stationary (OD_{600} > 8) growth phase. The *E. coli* K12 derivate strain DH5α [F′ Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK-, mK+) phoA supE44 λ– thy-1 gryA96 relA1] and GM2929 [F ara-14 leuB6 thi-1 hufA31 lacY1 tsx-78 galK2galT12glN44hisG4repsL136 (StrR) xylS-5 mtl-1 dam13::Tn9 (CamR) dcm-6 mcrB1 hsddR2 (rK– mK–) mcrA recF143] were grown as triplicates starting from a single colony each in 50 mL LB and incubated at 37 °C, 200 rpm until stationary growth phase. The number of bacterial and yeast cells were estimated on a CASY TT (Roche) cell counter. Yeast and bacterial cell pellets were centrifuged at 5000g for 3 min and washed with water before storage at −80 °C.

**DNA Extraction.** *A. thaliana* DNA was extracted from 100 mg leaf tissue of *Columba* 0 plants grown on a long day (16 h light, 8 h dark) for 3–4 weeks using a Plant Genomic DNA Miniprep Kit (GeneElute G2N70–1KT). *M. musculus* DNA was extracted from 15 mg of liver tissue obtained from female mice.
of mixed C57BL/6/SV129 background using the Genomic-tip 20/G kit (Qiagen). *D. melanogaster* DNA was extracted from a mixed population of 10 female and 10 male wt/w118 adult flies using a Gentra Puregene DNA purification kit (Qiagen). Yeast and bacterial DNA were extracted from 1.5 × 10⁹ or 4.5 × 10⁹ cells, respectively, using the Genomic-tip 20/G kit (Qiagen). DNA extracts were treated with RNase A at 37 °C for 45 min and DNA purification was performed according to the manufacturer’s instructions. Purified DNA was precipitated with isopropanol and washed with 70% ethanol and resuspended in 10 mM Tris-HCl, pH 8.0. DNA was quantified using dsDNA BR Assay Kit (Qubit) and quality controlled by gel electrophoresis.

**Sample Preparation for LC–MS/MS.** DNA samples were treated with DNA Degradase Plus (ZymoResearch, E2021) to manufacturer Josmar Langner for the (all University of Oxford) for an inspiring discussion. We thank Felix Krueger for help in the interpretation of the biSeq results. Work in the Ralser lab is funded by the Wellcome Trust (RG 093755/Z/10/Z) and the ERC (Starting Grant 260809). M.R. is a Wellcome Trust Research Career Development and Wellcome-Beit Prize fellow.

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**Author Contributions**

F.C. and M.M. established the LC-SRM method; F.C. prepared the DNA samples; F.C., M.M., and R.K. conducted the measurements; F.C., M.M., and M.R. analyzed the data; M.R. wrote the first manuscript draft; and all authors wrote the final manuscript.

**Notes**

The authors declare no competing financial interest.

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