White Rose Research Online

eprints@whiterose.ac.uk
https://eprints.whiterose.ac.uk
Universities of Leeds, Sheffield and York

Deposited via The University of York.

White Rose Research Online URL for this paper:
https://eprints.whiterose.ac.uk/id/eprint/116843/

Version: Accepted Version

Article:
Walters, Alison D and Chong, James P J (2017) Non-essential MCM-related proteins mediate a response to DNA damage in the archaeon Methanococcus maripaludis. Microbiology. pp. 1-9. ISSN: 1465-2080

https://doi.org/10.1099/mic.0.000460

Reuse
Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

UNIVERSITY OF LEEDS
University of Sheffield
UNIVERSITY of York

Microbiology

Non-essential MCM-related proteins mediate a response to DNA damage in the archaeon Methanococcus maripaludis

--Manuscript Draft--

Manuscript Number:	MIC-D-16-00444R2
Full Title:	Non-essential MCM-related proteins mediate a response to DNA damage in the archaeon Methanococcus maripaludis
Article Type:	Standard
Section/Category:	Physiology and metabolism
Corresponding Author:	James Chong University of York York, UNITED KINGDOM
First Author:	Alison D. Walters
Order of Authors:	Alison D. Walters
	James P.J. Chong
Abstract:	The single minichromosome maintenance (MCM) protein found in most archaea has been widely studied as a simplified model for the MCM complex that forms the catalytic core of the eukaryotic replicative helicase. Organisms of the order Methanococcales are unusual in possessing multiple MCM homologues. The Methanococcus maripaludis S2 genome encodes four MCM homologues, McmA - McmD. DNA helicase assays reveal that the unwinding activity of the three MCM-like proteins is highly variable despite sequence similarities and suggests additional motifs that influence MCM function are yet to be identified. While the gene encoding McmA could not be deleted, strains harbouring individual deletions of genes encoding each of the other MCMs display phenotypes consistent with these proteins modulating DNA damage responses. M. maripaludis S2 is the first archaeon in which MCM proteins have been shown to influence the DNA damage response.

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Manuscript Including References (Word document) Click here to download Manuscript Including References (Word document) Walters_Chong_20170302_final.docx

Non-essential MCM-related proteins mediate a response to DNA damage in the archaeon *Methanococcus maripaludis*

Alison D. Walters[1,2] and James P. J. Chong[1]*

[1]Department of Biology (Area 5), University of York, Wentworth Way, Heslington, York, YO10 5DD. UK.
[2]Current address: NIH/NIDDK, 8 Center Drive, Bethesda, MD20892, USA.

* corresponding author
Tel: +44 1904 328628
Fax: +44 1904 328505
Email: james.chong@york.ac.uk

Running title: MCM-mediated DNA damage response in archaea

Key words: MCM, DNA damage, DNA replication, methanogen

ABSTRACT

The single minichromosome maintenance (MCM) protein found in most archaea has been widely studied as a simplified model for the MCM complex that forms the catalytic core of the eukaryotic replicative helicase. Organisms of the order *Methanococcales* are unusual in possessing multiple MCM homologues. The *Methanococcus maripaludis* S2 genome encodes four MCM homologues, McmA – McmD. DNA helicase assays reveal that the unwinding activity of the three MCM-like proteins is highly variable despite sequence similarities and suggests additional motifs that influence MCM function are yet to be identified. While the gene encoding McmA could not be deleted, strains harbouring individual deletions of genes encoding each of the other MCMs display phenotypes consistent with these proteins modulating DNA damage responses. *M. maripaludis* S2 is the first archaeon in which MCM proteins have been shown to influence the DNA damage response.

INTRODUCTION

The eukaryotic minichromosome maintenance (MCM) complex comprises six homologous proteins, MCM2 – MCM7, all of which are required for DNA replication initiation and fork progression *in vivo*. MCM genes in eukaryotes have been demonstrated to be essential through the generation of temperature sensitive and degron mutants [1]. The MCMs appear to act as a nucleation point for the formation of the Cdc45-MCM-GINS (CMG) multi-protein complex necessary for DNA unwinding in eukaryotes [2]. Within the CMG complex, MCMs provide the replicative helicase activity required by eukaryotes during chromosomal DNA replication [3]. Unwinding activity in this complex is likely to be tightly controlled, as evidenced by the number of post-translational modifications reported for the MCM proteins [4-6]. The intracellular concentration of MCMs also has an important influence on the ability of cells to cope with replicative stress. Reduction of MCM concentrations reduces the ability of cells to cope with replicative challenges [7-9]. MCMs are a target of the ATM/ATR DNA damage checkpoint [10,11], which can be triggered by the Mre11-Rad50 complex binding to double-stranded DNA breaks [12,13]. Additional evidence suggests that the MCMs, in particular MCM3 [14], may directly influence DNA replication checkpoints to ensure replicative integrity [15-19], although the precise role MCMs play in the modulation of DNA repair pathways is still unclear. Other eukaryotic MCM paralogues have been shown to have a role in the repair of meiotic DNA breaks in mice [20], mammalian DNA mismatch repair [21] and the facilitation of DNA repair at homologous recombination sites [22].

Archaeal MCM homologues have been used as simplified models for understanding the mechanisms employed by the MCM complex in DNA unwinding [23]. Biochemical analysis of archaeal MCMs has led to the identification of a number of motifs that are essential for DNA binding, ATP hydrolysis and DNA helicase activities [24-26]. In all archaea studied to date, with the exception of *Thermococcus kodakarensis*, a single functional MCM has been identified that forms a homohexameric complex possessing these activities [27].

Members of the archaeal order *Methanococcales* possess between two and eight MCM homologues [28,29]. *Methanococcus maripaludis* S2 encodes four MCM homologues [28,30] corresponding to ORF numbers MMP0030, MMP0470, MMP0748 and

MMP1024. We have named these genes *mcmA*, *B*, *C* and *D* respectively [28]. Homologues of McmA and McmD are conserved in all *Methanococcales* species and appear to have arisen from an ancient duplication [28]. Phylogenetic analysis shows that the *M. maripaludis* MCMs are more closely related to one another than to MCMs from other archaea (Fig. 1(a)). While archaea with multiple MCMs have been identified outside the order *Methanococcales*, in most of these species there are truncations or mutations in residues that are essential for DNA helicase activity that result in the presence of only a single functional MCM protein [31,32]. An exception to this general observation is in *T. kodakarensis*, where the genome encodes three MCMs (MCM1-3), all of which are expressed, but only one of which (MCM3) is essential [33]. Deletion of *MCM1* or *MCM2* in *T. kodakarensis* did not affect cell growth or viability, indicating that they are non-essential for DNA replication [33]. As in *T. kodakarensis,* multiple sequence alignments of the *M. maripaludis* proteins with other archaeal proteins show that the motifs known to be required for MCM function are all conserved in McmA-D (Fig. 1(b)). Thus, all four of the *M. maripaludis* MCMs could potentially function as DNA helicases. McmD possesses additional amino acids between the second pair of cysteines within the zinc finger (Fig. 1(b)) and a C-terminal 20 amino acid insert, reminiscent of an insert observed in eukaryotic MCM3 [28]. The four *M. maripaludis* MCMs co-purify when co-expressed in *E. coli*, indicating that they can form heteromeric complexes *in vitro* [28]. *M. maripaludis* represents an interesting model for studying MCM function not only because it has multiple MCM homologues but, unusually for an archaeon, a well-established set of genetic tools are available for this organism [34] which allows both genetic and biochemical experiments to be used in the dissection of MCM function.

In this study we demonstrate that at least two of the four *M. maripaludis* MCMs (McmA and McmB) show robust DNA helicase activity *in vitro*. We have determined that only *mcmA* appears to be essential but that mutant strains deleted for non-essential MCMs show changes in cell cycle distribution and their responses to DNA damage. We have demonstrated that multiple MCM proteins are required for normal proliferation in this organism and that deletion of non-essential MCMs has significant effects on DNA damage responses.

METHODS

Sequence alignments and phylogenetics

Multiple sequence alignments were generated using ClustalX [35] and were used to construct a neighbour-joining tree.

Recombinant protein expression and purification

His-tagged proteins were expressed in Rosetta BL21(DE3) (Novagen) at 37 °C or Arctic Express (RIL) (Stratagene) at 12 °C. Expression was induced at 0.8 OD_{600nm} by 0.5 mM IPTG (final concentration). Cells were sonicated in lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 5% glycerol, 5 mM imidazole, 0.1 mM PMSF, 1 µg ml^{-1} pepstatin, 1 µg ml^{-1} leupeptin, 1 µg ml^{-1} aprotonin) with 0.75 mg ml^{-1} lysozyme and 5 µg ml^{-1} DNase. Lysate was clarified by centrifugation (50000 xg) and bound to 1 ml Talon beads (Clontech), washed with 10 column volumes (cv) of wash buffer (lysis buffer plus 10 mM imidazole) and protein was eluted in elution buffer (lysis buffer plus 150 mM imidazole). Fractions were pooled, diluted 1:3 in dilution buffer (10 mM Tris pH 8.0, 5% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 0.1% β-mercaptoethanol) and loaded on a 1 ml Source Q column (GE Healthcare), washed with 10 cv start buffer and eluted over a 20 cv gradient to 500 mM NaCl. Elution fractions were analysed by SDS-PAGE and concentrated into 10 mM Tris pH 7.5.

Strand displacement assays

Forked substrate DNA was prepared by γ-^{32}P labelling oligo HS2 (5'-TTTGTTTGTTTGTTTGTTTGTTTGTTTGCCGACGTGCCAGGCCGACGCGTCCC-3') and annealing to HS1 (5'GGGACGCGTCGGCCTGGCACGTCGGCCGCTGCGGCCAGGCACCCGATGGCGTTTGTTTGTTTGTTTGTTTGTTT-3') as described [36]. A 10 µl reaction containing HDB [27], 2.5 mM ATP, 150 mM potassium glutamate and 1 nM labelled substrate was prepared on ice. 10 µl protein aliquots (0-2400 fmol hexamer) in 50 mM potassium glutamate, 10 mM HEPES pH 7.6 were prepared on ice. 10 µl of the reaction mix was added to each protein aliquot and incubated at 37 °C for 1 hour. Substrate alone was boiled for 5 minutes then placed on ice. Reactions were stopped by the addition of 5 µl 200 mM EDTA, 1% SDS, 20% glycerol, 0.4 pmol μl^{-1} unlabelled HS2 oligo, 1 µg μl^{-1} proteinase K. DNA was separated on 12% native polyacrylamide gels, dried and visualised using a phosphorimager (BioRad). Results were quantified using Quantity

143 One software (BioRad).
144
145 **Markerless mutagenesis in *M. maripaludis* S2**
146 Genetic manipulations were carried out using the Mm900 (S2 *Δhpt*) strain of *M.*
147 *maripaludis* [37]. Deletion plasmids were constructed by cloning 500 bp of upstream and
148 downstream flanking DNA into the *Not* I site of pCRPrtNeo including codons for the five
149 N-terminal and C-terminal amino acids of each MCM to ensure read-through
150 (oligonucleotide sequences available on request) [37]. Transformations and markerless
151 mutagenesis were carried out as described [37]. New strains were streak-purified,
152 screened by PCR and analysed by Southern blot.
153
154 **Southern blots**
155 Southern blotting was carried out using DIG-labelling and detection kit according to
156 manufacturer's instructions (Roche). Genomic DNA from individual strains was digested
157 with the following restriction enzymes to generate appropriate fragments for probing:
158 mcmA (*Pst* I), mcmB (*Sac* I, *Pvu* II), mcmC (*Pst* I, *Sac* I), mcmD (*Nci* I, *Xho* I). Regions
159 of interest were detected using digoxin random hexamer-labelled probes to 500 bp
160 flanking regions of each MCM (Fig. S1). Blots were visualised by CPSD detection
161 (Roche) and exposing to photographic film for 1-5 minutes.
162
163 **Culture and cell sampling of *M. maripaludis***
164 *M. maripaludis* was cultured in McCas liquid media as described [37]. For batch culture
165 of *M. maripaludis*, 2 litres of modified McCas medium was prepared in a sealed 3 litre
166 bioreactor (Applikon Ltd.) as previously described [38]. The medium was inoculated
167 using 5x 5 ml cultures of *M. maripaludis* at an OD_{600nm} of 0.7-1.0. After inoculation,
168 optical density was measured at 600nm every 2-5 hours. Sodium dithionite was added to
169 samples before OD_{600nm} was measured aerobically.
170
171 **Flow cytometry**
172 1 ml of *M. maripaludis* culture was centrifuged (16000 xg, 5 minutes, room temperature).
173 The pellet was resuspended in 100 μl of TSE buffer (10 mM Tris pH 7.5, 10 mM EDTA,
174 380 mM NaCl, 200 mM KCl). 1 ml ice cold (77% ethanol, 600 mM LiCl) was added, the
175 sample was vortexed then stored at 4 °C. Before analysis, fixed cells were pelleted
176 (16000 xg, 5 minutes, room temperature), resuspended in 1 ml buffer A (10 mM Tris pH

7.5, 10 mM $MgCl_2$), spun and then resuspended in 150 μl buffer A containing 100 μg ml^{-1} mithramycin A / 20 μg ml^{-1} ethidium bromide. Stained cells were analysed by Apogee A40 MiniFCM with a 50 mW 405 nm laser. 100,000-500,000 cells were analyzed for each sample. Data were processed using FlowJo (Treestar).

DNA damage

DNA damage assays were conducted under strict anaerobic conditions. For UV damage assays, 10^8-10^9 cells were diluted in McCas medium and spotted on McCas plates. Spots were air dried and then exposed to UV (254 nm). Post-treatment, plates were shielded from visible light. UV dosage was measured using a Blak-Ray UV meter (UVP, Inc). For ionising radiation damage assays, aliquots of cultures were exposed to a calibrated X-ray dose from an X-ray generator. After exposure to X-rays, 10^8-10^9 cells were diluted in McCas medium and spotted on McCas plates. Plates were pressurised to 20 PSI with a 4:1 ratio of H_2:CO_2 and then incubated at 37 °C for 5 days.

RESULTS

McmA and McmB display in vitro DNA helicase activity

To investigate whether individual MCMs possessed DNA helicase activity, hexa-histidine-tagged recombinant McmA, McmB and McmC were purified using affinity and anion exchange chromatography (Fig. 2(a)). McmD was largely insoluble when expressed recombinantly, even when protein folding was facilitated by the presence of *Oleispira antarctica* chaparones Cpn10 and Cpn60 at 12°C. Size exclusion chromatography of soluble Mcms A-C under different salt conditions support the notion that these complexes might form a range of multimeric complexes in solution (Fig. S2). Walker A motif lysine to glutamate (K>E) mutants were expressed and purified in the same manner and used as negative controls in DNA helicase assays (Fig. 2(b)-(d)). The helicase activity of individual MCMs was tested using a strand displacement assay with a forked substrate containing a 25 bp double-stranded region [36]. Both McmA and McmB showed protein concentration-dependent helicase activity (Fig. 2(b),(c)). The unwinding activity of McmB at the highest protein concentration (82% of substrate) was slightly higher than that of McmA (77% of double stranded substrate). However, McmB displayed considerably higher DNA unwinding rates than McmA at lower protein concentrations (Fig. 2(e)). In contrast, we were unable to detect any significant DNA helicase activity in McmC over the same range of concentrations (Fig. 2(d)).

McmA is essential

In order to ascertain whether any of the *M. maripaludis* MCMs were essential, deletions of each of the four individual MCMs were undertaken using a markerless mutagenesis strategy [37]. Genomic DNA was isolated from the resulting strains and analysed by Southern blotting to confirm whether a deletion mutant could be generated for each MCM gene. Deletion mutants were isolated for *mcmB*, *mcmC* and *mcmD*, demonstrating that these three genes are non-essential (Fig. 3(b)-(d)). We were unable to isolate a *mcmA* deletion strain despite screening more than 75 colonies from three independent transformations, consistent with the hypothesis that this gene is essential (Fig. 3(a)). This observation is supported by a recent genome-wide transposon mutagenesis study in *M. maripaludis* that classified McmA as "possibly essential" [39].

Deletion of non-essential MCMs results in proliferation defects

We generated growth curves for each of the *Δmcm* strains from batch cultures grown in a 3 litre anaerobic fermenter to compare to WT (Mm900, Fig. 4(a),(b)), [37]. In all cases doubling times of the *Δmcm* strains were shorter than WT, although specific growth rates and doubling times of *ΔmcmB* and *ΔmcmD* were very similar to those calculated for WT (Table 1). *ΔmcmC* displayed an obvious decrease in calculated doubling time compared to WT of ~20% (Table 1). Lag phases for all *Δmcm* strains were longer than observed for WT (Fig. 4(a)). Further experiments are required to understand this phenomenon.

DNA content and cell size for samples taken throughout the growth period were analysed by flow cytometry (Fig. 4(c)-(e)) and compared between WT and *Δmcm* cells at similar optical densities across the entire growth range. The cell cycle distribution of *M. maripaludis* is similar to that observed for *Methanocaldococcus jannaschii* [40]. *M. maripaludis* cells show a broad distribution of DNA content and cell size, with no distinct genome peaks visible during exponential growth in contrast to the distinct genome peaks observed for *Archaeoglobus fulgidus, Methanothermobacter thermautotrophicus* and *Sulfolobus solfataricus* [32,40,41]. This observation supports the previous observation [42], that *M. maripaludis* cells are highly polyploid under normal growth conditions, as is the case for exponentially growing bacteria [43] and halophilic archaea [44].

Although some consistent minor differences between WT and *ΔmcmB or ΔmcmC cells* were observed, overall these deletions appeared to have no significant effects on cell size or DNA content compared to WT (Fig. 4(c), (d)). *ΔmcmD* cells were larger than WT in all growth phases. *ΔmcmD* cells also possessed a greater DNA content than WT in early and mid-log growth (Fig. 4(e)). *ΔmcmD* cells with a very low DNA content increased dramatically in late log/stationary phase to become the dominant population. This phenotype could be indicative of DNA breakage, perhaps caused by incomplete DNA replication, aberrant DNA segregation, defective cell division or an inability to effectively repair DNA damage accumulated during growth.

MCMs mediate a DNA damage response

To determine whether the *ΔmcmD* cell cycle distribution differences we observed were due to a defect in the ability of these cells to respond to DNA damage, we subjected WT and mutant strains to increasing doses of UV radiation. Consistent with previous reports [45] we found *M. maripaludis* S2 cells to be highly sensitive to UV damage (Fig. 5(a)). This sensitivity was dramatically increased in *ΔmcmD* but slightly reduced in both *ΔmcmB* and *ΔmcmC*, which were more resistant to low doses of UV damage than WT. These phenotypes were confirmed by exposing the same strains to ionising radiation, where *ΔmcmD* also showed hypersensitivity this type of damage (Fig. 5(b)). Consistent with our observations for UV damage, *ΔmcmB* and *ΔmcmC* showed an increased resistance to ionising radiation compared to WT (Fig. 5(b)).

DISCUSSION

We have produced recombinant protein for three highly similar McmA-type MCMs from *M. maripaludis* S2. McmA and McmB displayed DNA helicase activity but McmC did not. Interestingly, although measurements by size exclusion chromatography shows complexes of different sizes under different conditions for McmA and McmB, they were still able to unwind DNA. This situation is similar to that described for the eukaryotic MCMs where a complex of MCMs 4, 6 and 7 is sufficient for *in vitro* helicase activity (probably as a dimer of trimers), but the active complex *in vivo* is additionally modulated by the presence of other MCM subunits [46]. *M. maripaludis* encodes multiple RecJ homologues, several of which have been shown to be non-essential, and a single GINS protein, which is probably essential [39]. We have previously reported the recovery of a

277 complex containing all four recombinant *M. maripaludis* Mcm proteins, supporting the
278 notion that a heteromeric complex may be formed *in vivo* [28]. It is also possible that
279 more than one Mcm complex is formed *in vivo*, providing different functions. The
280 absence of helicase activity in McmC and the faster unwinding rate of McmB suggest
281 that additional amino acids to those already identified in the MCM proteins are critical for
282 modulating helicase activity in complexes formed by individual proteins. A detailed
283 analysis of the McmC sequence compared to McmA/McmB could provide important
284 insights into the modulation of MCM helicase activity and the molecular mechanisms
285 governing this activity in eukaryotes.
286
287 Our results demonstrate that *M. maripaludis* possesses multiple functional MCMs, one of
288 which is essential, with the other three causing defects in cell proliferation and the
289 response to DNA damage when deleted. *mcmA* could not be deleted and displays
290 robust helicase activity *in vitro*. McmB had more vigorous DNA helicase activity than
291 McmA *in vitro* and when deleted, increased resistance to DNA damage. Δ*mcmC*
292 displayed a faster growth rate than WT and increased resistance to DNA damage. In
293 contrast, Δ*mcmD* showed a striking increase in DNA damage sensitivity. A previous
294 shotgun proteomics study detected peptides for McmA, McmB and McmD *in vivo* [47].
295 These data support our findings that McmB and McmD have functional roles *in vivo*.
296 While peptides for McmC were not detected, this does not definitively prove that such
297 peptides were not present. We have been unable to obtain sufficient soluble McmD to
298 conduct helicase assays, so whether McmD is an active helicase remains unknown. Our
299 previous genome context analysis revealed an upstream ORF of unknown function that
300 is likely to be operonic with *mcmD* in *M. maripaludis* S2 [28]. Interestingly, this ORF is
301 highly conserved throughout the *Methanococcales* (Fig. S3, S4), but not found in any
302 other species. The positioning of this ORF contiguous with *mcmD* is conserved among
303 the mesophilic *Methanococcales.* It is possible that co-expression of this smaller ORF
304 with McmD would produce soluble protein to allow biochemical analysis.
305
306 We have previously noted that McmD possesses a modified zinc finger and C-terminal
307 20 amino acid insert and similar features are found in eukaryotic MCM3 [28]. MCM3 has
308 been implicated in the regulation of the eukaryotic MCM complex [4], and has been
309 shown to be a specifically phosphorylated by ATM/ATR kinases [10]. An apparent
310 requirement for the specific proteolysis of eukaryotic MCM3 before apoptosis can be

311 induced has also been reported [48,49]. Thus the notion of a specialised Mcm as a
312 nexus for a modulatory or checkpoint decision is not without precedent. The response of
313 *ΔmcmD* to UV and ionizing radiation supports the notion that McmD is important either in
314 modulating a response to DNA damage or that McmD is important in controlling the
315 polyploidy observed in *M. maripaludis*, which in turn could influence the cell's ability to
316 repair damage through homologous recombination pathways as reported for
317 *Deinococcus*. The altered cell size and DNA content of *ΔmcmD* measured using flow
318 cytometry, supports the hypothesis that McmD may have a role in proliferation control.
319
320 *ΔmcmB* or *ΔmcmC* strains are more resistant to DNA damage than WT. This response is
321 reminiscent of phenotype observed in polyploid *Haloferax volcanii* when the DNA repair
322 genes *mre11* and *rad50* are deleted [50]. It has been suggested the Mre11-Rad50
323 complex delays the repair of damage by homologous recombination to allow DNA repair
324 to occur more rapidly using microhomology mediated end-joining, avoiding the
325 complications inherent in using homologous recombinational repair in a polyploid
326 organism. *H. volcanii mre11 rad50* mutants therefore undergo homologous repair more
327 readily than WT, enhancing cell survival but reducing the recovery rate from DNA
328 damage [50]. *ΔmcmB* or *ΔmcmC* strains may bypass the preferred DNA damage
329 response to similarly undergo homologous recombination to repair DNA damage.
330 Whether the DNA repair processes that take place under these circumstances are error-
331 prone or error-free and whether the long-term fitness of *ΔmcmB* or *ΔmcmC* strains is
332 reduced remains to be determined.
333
334 The responses to deletion of MCM genes in *M. maripaludis* have allowed us to clearly
335 describe the first example of an archaeal organism where MCMs play a role in the
336 response to DNA damage. This observation indicates that, as in eukaryotes, the multiple
337 MCMs in *M. maripaludis* have evolved to perform specialized functions. Interestingly,
338 protein interaction studies in *T. kodakarensis* show that non-essential MCM1 and MCM2
339 co-purify with proteins with known roles in DNA repair [51], although a role for these
340 MCMs in DNA repair has not be established. Our data demonstrating that multiple
341 functional MCMs are present in *M. maripaludis* indicate that this organism provides a
342 useful biochemical and genetic system that could provide further insight into eukaryotic
343 MCM function.
344

FUNDING INFORMATION

The Worldwide Universities Network provided travel grant funds to A.D.W. This work was supported in part by a Biotechnology and Biological Sciences Research Council PhD studentship. J.P.J.C. is a Royal Society Industry Fellow.

ACKNOWLEDGEMENTS

Thanks to John Leigh and Tom Lie for providing strains, plasmids and expertise in *M. maripaludis* genetics, Jo Milner for the loan of the UV dosimeter.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

357 REFERENCES

358 1. **Labib K, Tercero JA, Diffley JF.** Uninterrupted MCM2-7 function required for
359 DNA replication fork progression. *Science* 2000;288:1643–1647.

360 2. **Ilves I, Petojevic T, Pesavento JJ, Botchan MR.** Activation of the MCM2-7
361 helicase by association with Cdc45 and GINS proteins. *Molecular Cell*
362 2010;37:247–258.

363 3. **Bochman ML, Schwacha A.** The Mcm complex: unwinding the mechanism of a
364 replicative helicase. *Microbiol. Mol. Biol. Rev.* 2009;73:652–683.

365 4. **Takei Y, Assenberg M, Tsujimoto G, Laskey R.** The MCM3 acetylase MCM3AP
366 inhibits initiation, but not elongation, of DNA replication via interaction with MCM3.
367 *J. Biol. Chem.* 2002;277:43121–43125.

368 5. **Sheu Y-J, Stillman B**. Cdc7-Dbf4 phosphorylates MCM proteins via a docking
369 site-mediated mechanism to promote S phase progression. *Molecular Cell*
370 2006;24:101–113.

371 6. **Lei M, Kawasaki Y, Young MR, Kihara M, Sugino A *et al*.** Mcm2 is a target of
372 regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. *Genes Dev.*
373 1997;11:3365–3374.

374 7. **Ibarra A, Schwob E, Méndez J.** Excess MCM proteins protect human cells from
375 replicative stress by licensing backup origins of replication. *Proc. Natl. Acad. Sci.*
376 *U.S.A.* 2008;105:8956–8961.

377 8. **Woodward AM, Göhler T, Luciani MG, Oehlmann M, Ge X *et al*.** Excess Mcm2-
378 7 license dormant origins of replication that can be used under conditions of
379 replicative stress. *J. Cell Biol.* 2006;173:673–683.

380 9. **Maki K, Inoue T, Onaka A, Hashizume H, Somete N *et al*.** Abundance of
381 prereplicative complexes (Pre-RCs) facilitates recombinational repair under
382 replication stress in fission yeast. *Journal of Biological Chemistry*
383 2011;286:41701–41710.

384 10. **Cortez D, Glick G, Elledge SJ.** Minichromosome maintenance proteins are direct
385 targets of the ATM and ATR checkpoint kinases. *Proc. Natl. Acad. Sci. U.S.A.*
386 2004;101:10078–10083.

387 11. **Shi Y, Dodson GE, Mukhopadhyay PS, Shanware NP, Trinh AT *et al*.**
388 Identification of carboxyl-terminal MCM3 phosphorylation sites using polyreactive
389 phosphospecific antibodies. *J. Biol. Chem.* 2007;282:9236–9243.

390 12. **Lee J-H, Paull TT.** ATM activation by DNA double-strand breaks through the
391 Mre11-Rad50-Nbs1 complex. *Science* 2005;308:551–554.

392 13. **D'Amours D, Jackson SP.** The Mre11 complex: at the crossroads of dna repair
393 and checkpoint signalling. *Nat. Rev. Mol. Cell Biol.* 2002;3:317–327.

394 14. **Han X, Pozo FM, Wisotsky JN, Wang B, Jacobberger JW *et al*.**

Phosphorylation of mini-chromosome maintenance 3 (MCM3) by Chk1 negatively regulates DNA replication and checkpoint activation. *Journal of Biological Chemistry* 2015;289:24716-24723.

15. **Komata M, Bando M, Araki H, Shirahige K.** The direct binding of Mrc1, a checkpoint mediator, to Mcm6, a replication helicase, is essential for the replication checkpoint against methyl methanesulfonate-induced stress. *Mol. Cell.* Biol. 2009;29:5008–5019.

16. **Bailis JM, Luche DD, Hunter T, Forsburg SL.** Minichromosome maintenance proteins interact with checkpoint and recombination proteins to promote s-phase genome stability. *Mol. Cell. Biol.* 2008;28:1724–1738.

17. **Trenz K, Smith E, Smith S, Costanzo V.** ATM and ATR promote Mre11 dependent restart of collapsed replication forks and prevent accumulation of DNA breaks. *EMBO J.* 2006;25:1764–1774.

18. **Han X, Aslanian A, Fu K, Tsuji T, Zhang Y.** The interaction between checkpoint kinase 1 (Chk1) and the minichromosome maintenance (MCM) complex is required for DNA damage-induced Chk1 phosphorylation. *Journal of Biological Chemistry* 2014;289:24716–24723.

19. **Ilves I, Tamberg N, Botchan MR.** Checkpoint kinase 2 (Chk2) inhibits the activity of the Cdc45/MCM2-7/GINS (CMG) replicative helicase complex. *Proc. Natl. Acad. Sci. U.S.A.* 2012;109:13163–13170.

20. **McNairn AJ, Rinaldi VD, Schimenti JC.** Repair of Meiotic DNA Breaks and Homolog Pairing in Mouse Meiosis Requires a Minichromosome Maintenance (MCM) Paralog. *Genetics* 2017;205:529–537.

21. **Park J, Long DT, Lee KY, Abbas T, Shibata E *et al.*** The MCM8-MCM9 complex promotes RAD51 recruitment at DNA damage sites to facilitate homologous recombination. *Mol. Cell. Biol.* 2013;33:1632–1644.

22. **Traver S, Coulombe P, Peiffer I, Hutchins JRA, Kitzmann M *et al.*** MCM9 Is Required for Mammalian DNA Mismatch Repair. *Molecular Cell* 2015;59:831–839.

23. **Bell SD, Botchan MR.** The minichromosome maintenance replicative helicase. *Cold Spring Harb Perspect Biol* 2013;5:a012807.

24. **McGeoch AT, Trakselis MA, Laskey RA, Bell SD.** Organization of the archaeal MCM complex on DNA and implications for the helicase mechanism. *Nat. Struct. Mol. Biol.* 2005;12:756–762.

25. **Jenkinson ER, Chong JPJ.** Minichromosome maintenance helicase activity is controlled by N- and C-terminal motifs and requires the ATPase domain helix-2 insert. *Proc. Natl. Acad. Sci. U.S.A.* 2006;103:7613–7618.

26. **Kasiviswanathan R, Shin J-H, Melamud E, Kelman Z.** Biochemical characterization of the *Methanothermobacter thermautotrophicus* minichromosome maintenance (MCM) helicase N-terminal domains. *J. Biol. Chem.*

2004;279:28358–28366.

27. **Chong JP, Hayashi MK, Simon MN, Xu RM, Stillman B.** A double-hexamer archaeal minichromosome maintenance protein is an ATP-dependent DNA helicase. *Proc. Natl. Acad. Sci. U.S.A.* 2000;97:1530–1535.

28. **Walters AD, Chong JPJ.** An archaeal order with multiple minichromosome maintenance genes. *Microbiology* 2010;156:1405–1414.

29. **Krupovic M, Gribaldo S, Bamford DH, Forterre P.** The evolutionary history of archaeal MCM helicases: a case study of vertical evolution combined with hitchhiking of mobile genetic elements. *Mol. Biol. Evol.* 2010;27:2716–2732.

30. **Hendrickson EL, Kaul R, Zhou Y, Bovee D, Chapman P *et al*.** Complete genome sequence of the genetically tractable hydrogenotrophic methanogen *Methanococcus maripaludis*. *J. Bacteriol.* 2004;186:6956–6969.

31. **McGeoch AT, Bell SD.** Extra-chromosomal elements and the evolution of cellular DNA replication machineries. *Nat Rev Mol Cell Biol* 2008;9:569–574.

32. **Majerník AI, Lundgren M, McDermott P, Bernander R, Chong JPJ.** DNA content and nucleoid distribution in *Methanothermobacter thermautotrophicus*. *J. Bacteriol.* 2005;187:1856–1858.

33. **Pan M, Santangelo TJ, Li Z, Reeve JN, Kelman Z.** *Thermococcus kodakarensis* encodes three MCM homologs but only one is essential. *Nucleic Acids Research* 2011;39:9671–9680.

34. **Sarmiento FB, Leigh JA, Whitman WB.** Genetic systems for hydrogenotrophic methanogens. *Meth. Enzymol.* 2011;494:43–73.

35. **Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG.** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 1997;25:4876–4882.

36. **Shin J-H, Jiang Y, Grabowski B, Hurwitz J, Kelman Z.** Substrate requirements for duplex DNA translocation by the eukaryal and archaeal minichromosome maintenance helicases. *J. Biol. Chem.* 2003;278:49053–49062.

37. **Moore BC, Leigh JA.** Markerless mutagenesis in *Methanococcus maripaludis* demonstrates roles for alanine dehydrogenase, alanine racemase, and alanine permease. *J. Bacteriol.* 2005;187:972–979.

38. **Haydock AK, Porat I, Whitman WB, Leigh JA.** Continuous culture of *Methanococcus maripaludis* under defined nutrient conditions. *FEMS Microbiol. Lett.* 2004;238:85–91.

39. **Sarmiento F, Mrázek J, Whitman WB.** Genome-scale analysis of gene function in the hydrogenotrophic methanogenic archaeon *Methanococcus maripaludis.* *Proc. Natl. Acad. Sci. U.S.A.* 2013;110:4726–4731.

40. **Bernander R, Poplawski A.** Cell cycle characteristics of thermophilic archaea. *J. Bacteriol.* 1997;179:4963–4969.

41. **Maisnier-Patin S, Malandrin L, Birkeland N-K, Bernander R.** Chromosome replication patterns in the hyperthermophilic euryarchaea *Archaeoglobus fulgidus* and *Methanocaldococcus (Methanococcus) jannaschii. Mol. Microbiol.* 2002;45:1443–1450.

42. **Hildenbrand C, Stock T, Lange C, Rother M, Soppa J.** Genome copy numbers and gene conversion in methanogenic archaea. *J. Bacteriol.* 2011;193:734–743.

43. **Cooper S, Helmstetter CE.** Chromosome replication and the division cycle of *Escherichia coli*. *Journal of Molecular Biology* 1968;31:519–540.

44. **Breuert S, Allers T, Spohn G, Soppa J.** Regulated polyploidy in halophilic archaea. *PLoS ONE* 2006;1:e92.

45. **Kiener A, Gall R, Rechsteiner T, Leisinger T.** Photoreactivation in *Methanobacterium thermautotrophicum*. *Archives of Microbiology* 1985;143:147–150.

46. **Ishimi Y.** A DNA helicase activity is associated with an MCM4, -6, and -7 protein complex. *J. Biol. Chem.* 1997;272:24508–24513.

47. **Xia Q, Hendrickson EL, Zhang Y, Wang T, Taub F *et al*.** Quantitative proteomics of the archaeon *Methanococcus maripaludis* validated by microarray analysis and real time PCR. *Mol. Cell Proteomics* 2006;5:868–881.

48. **Schwab BL, Leist M, Knippers R, Nicotera P.** Selective proteolysis of the nuclear replication factor MCM3 in apoptosis. *Exp. Cell Res.* 1998;238:415–421.

49. **Schories B, Engel K, Dörken B, Gossen M, Bommert K.** Characterization of apoptosis-induced Mcm3 and Cdc6 cleavage reveals a proapoptotic effect for one Mcm3 fragment. *Cell Death Differ.* 2004;11:940–942.

50. **Delmas S, Shunburne L, Ngo H-P, Allers T.** Mre11-Rad50 promotes rapid repair of DNA damage in the polyploid archaeon *Haloferax volcanii* by restraining homologous recombination. *PLoS Genet* 2009;5:e1000552.

51. **Li Z, Santangelo TJ, Cuboňová L, Reeve JN, Kelman Z.** Affinity purification of an archaeal DNA replication protein network. *MBio* 2010;1.

Table 1

Growth rates of Mm900 (wild type) and *Δmcm* strains calculated from Fig. 4(b).

Strain	Specific growth rate (μ)	Doubling time (hours) $T_2=\ln 2/\mu$
Mm900 (WT)	0.0029	3.98
ΔmcmB	0.0032	3.61
ΔmcmC	0.0036	3.20
ΔmcmD	0.0030	3.85

FIGURE LEGENDS

Figure 1

Multiple potentially functional MCMs in *M. maripaludis*.

(a) The *Methanococcus maripaludis* MCMs are more related to each other than to other archaeal MCMs. Phylogenetic tree of *M. maripaludis* MCMs (Mmp) compared to MCM sequences from *Methanothermobacter thermautotrophicus* (Mth), *Archaeoglobus fulgidus* (Afu), *Sulfolobus solfataricus* (Sso), *Aeropyrum pernix* (Ape) and *Korarchaeum cryptophilum* (Kcr).

(b) *M. maripaludis* MCMs appear to contain all the sequence motifs known to be required for helicase activity. Alignment of the sequences used in (a) in the same order showing conservation of motifs and essential residues that have been experimentally determined to be required for helicase activity. The helix-2 insert (h2-i) is not conserved at amino acid level, but is present in all sequences and shown as a box. Catalytically important amino acids are shown in bold, residues that deviate from typical motifs, but are known to support function are shaded.

Figure 2

Biochemical characterisation of MCMs in *M. maripaludis*.

(a) SDS-PAGE gels showing purified recombinant McmA, B and C proteins after affinity (Co^{2+}) and anion exchange (AX) chromatography. (b) strand displacement assay for McmA. Protein concentrations are indicated in fmol hexamer. K>E indicates Walker A mutant of McmA (1200 fmol hexamer), -ATP is wild type protein (1200 fmol hexamer) in the absence of ATP. (c) strand displacement assay for McmB. Lanes and protein

concentrations are as indicated for (b). (d) strand displacement assay for McmC. Lanes and protein concentrations are as indicated for (b). (e) quantification of strand displacement activities for McmA (closed circles), McmB (open circles) and McmC (crosses), representative data were acquired from the figures in (b)-(d). Each experiment was repeated at least three times.

Figure 3

Three of the four MCMs in *M. maripaludis* can be deleted.

The Mm900 (WT) strain was subjected to markerless mutagenesis (Moore and Leigh, 2005) to delete MCM genes. Strains were recovered and subjected to Southern blot to confirm whether deletion strains could be generated. In all cases, lane 1 contains molecular weight markers, lane 2 WT genomic DNA, lane 3 the relevant merodiploid to show that the mutagenesis was successful. (a) no deleted strains of *mcmA* were recovered. Lanes 4-23 are WT strains recovered from markerless mutagenesis. (b) *ΔmcmB* strains were identified in lanes 13, 21 and 23. (c) *ΔmcmC* strains were identified in lanes 4, 6, 8, 12, 16 and 21. (d) *ΔmcmD* deleted strains were identified in lanes 8, 11 and 20.

Figure 4

MCM deletions result in proliferation defects.

(a) Time course measurements of OD_{600} as an indication of cell number. WT (Mm900, closed circles) or *M. maripaludis* strains harbouring deletions in *mcmB* (open circles), *mcmC* (closed squares) or *mcmD* (open squares) were grown in a 2L batch culture and sampled as indicated. (b) Exponential growth data from (a) replotted as $\ln(OD_{600})$ for the calculation of doubling times (see Table 1). Symbols as for (a), regressions shown as grey dotted lines. (c) - (e) Flow cytometry indicates that deletion of non-essential MCMs in *M. maripaludis* results in a proliferation phenotype. (c) *ΔmcmB*, (d) *ΔmcmC*, (e) *ΔmcmD*. In each panel the profile for WT cells at a similar OD_{600} is shown in grey, the MCM deleted strain profile is shown as a black line. Discontinuities at the mid-point in each curve are due to automatic switching between different photomultipliers for detection of small signals in the Apogee flow cytometer used to make these measurements. Within each group of panels, the left column panels show light scatter as an indication of cell size; the right column panels show fluorescence as an indication of

DNA content. Event number is normalized. Data are plotted on a logarithmic scale. Numbers indicate the OD_{600} of deletion strain (top) compared to wild type (bottom).

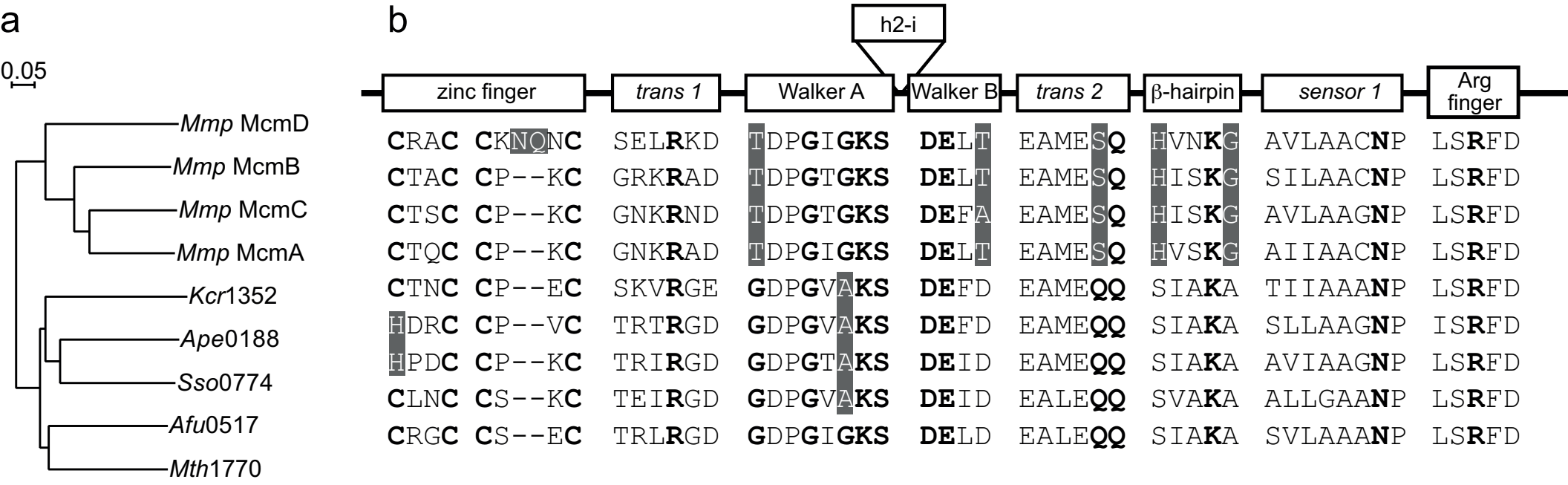
Figure 5

Δmcm strains show DNA damage phenotypes.

(a) WT *M. maripaludis* (Mm900, closed circles), *ΔmcmB* (diamonds), *ΔmcmC* (triangles) or *ΔmcmD* (open circles) strains were plated at different dilutions before being irradiated with UV light (254 nm) as indicated. Surviving cells were calculated by enumerating colonies formed. The mean and standard errors for three independent experiments are shown. *ΔmcmB* and *ΔmcmC* strains are more resistant to low UV doses than WT, whereas *ΔmcmD* is more sensitive to this type of damage. (b) The same strains, indicated by the same symbols as (a) were subjected to ionizing radiation (X-rays) as indicated. *ΔmcmD* was substantially more sensitive to DNA damage than WT or the *ΔmcmB* and *ΔmcmC* strains, which were more resistant to damage. The mean and standard errors for three independent experiments are shown.

Figure 1

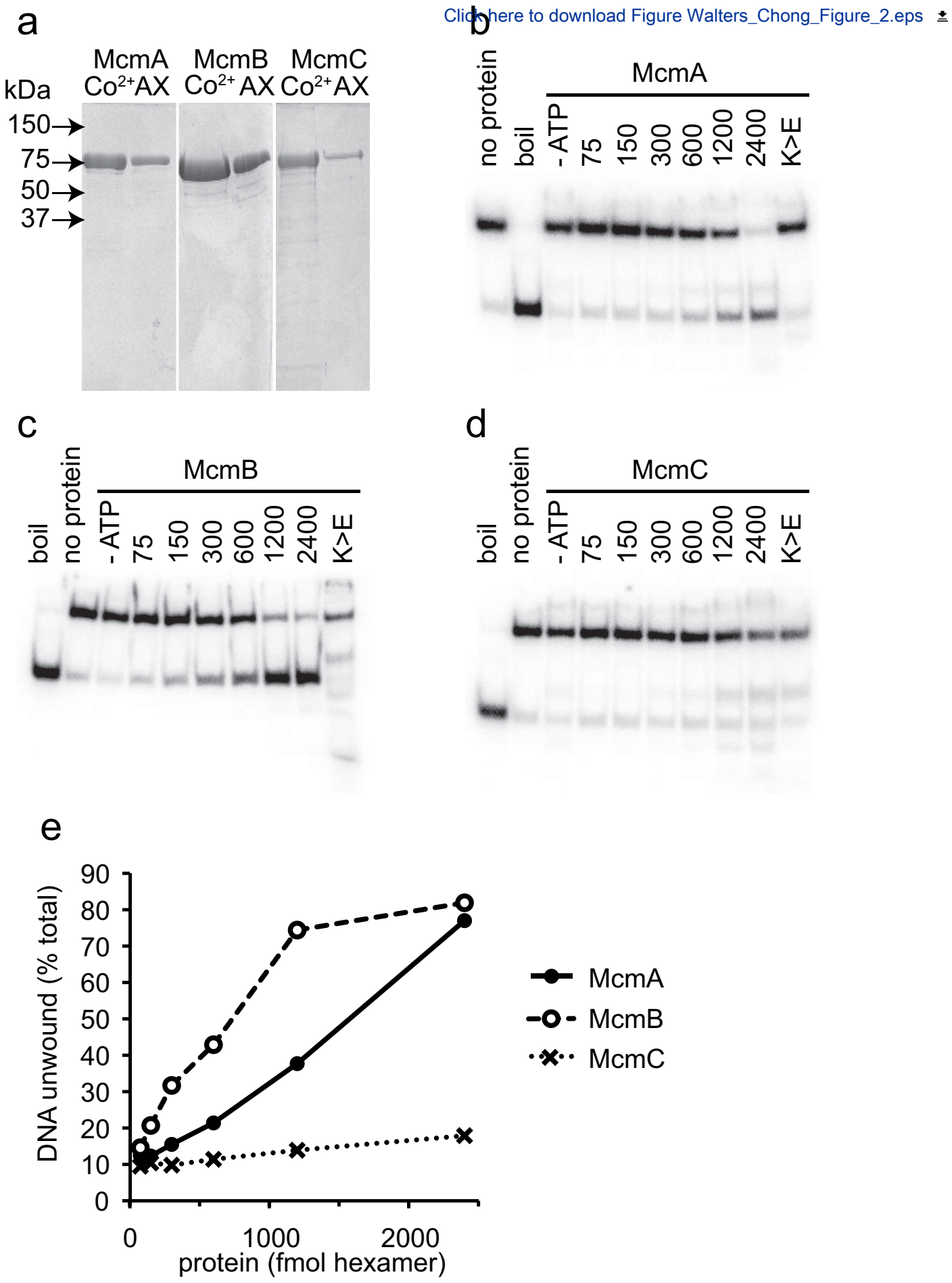
Click here to download Figure Walters_Chong_Figure_1.eps



Walters and Chong - Figure 1

Figure 2

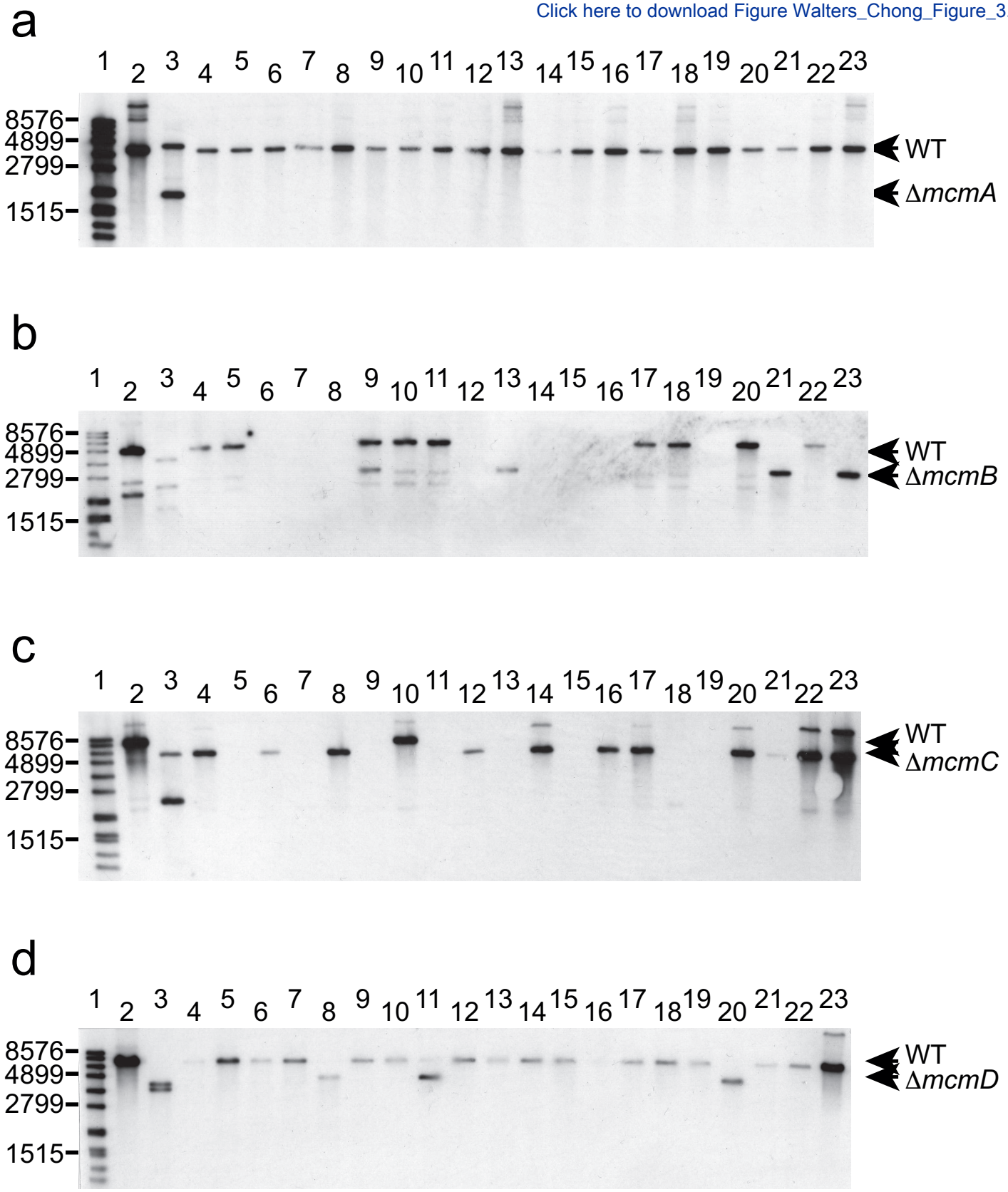
Click here to download Figure Walters_Chong_Figure_2.eps



Walters and Chong - Figure 2

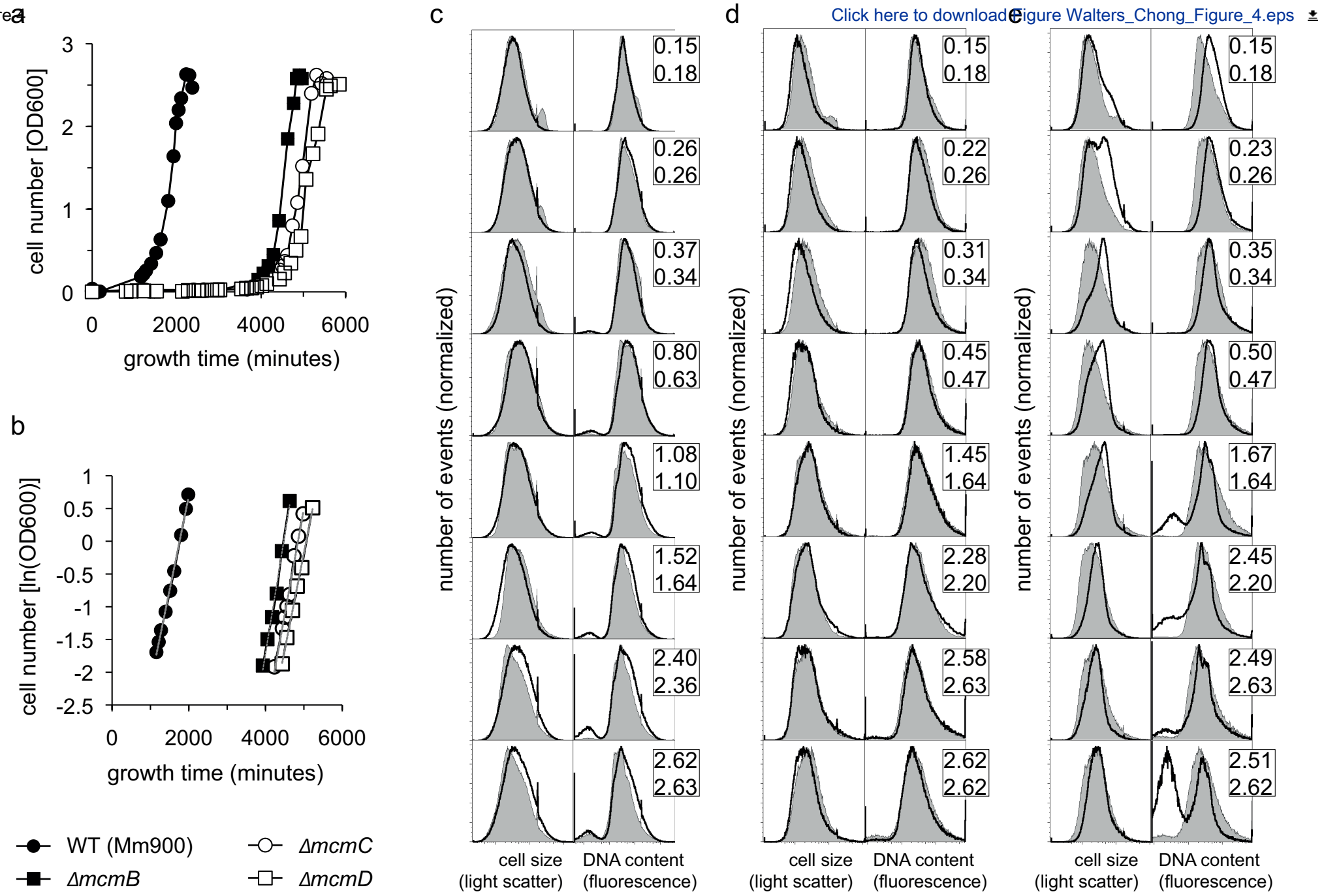
Figure 3

Click here to download Figure Walters_Chong_Figure_3.eps



Walters and Chong - Figure 3

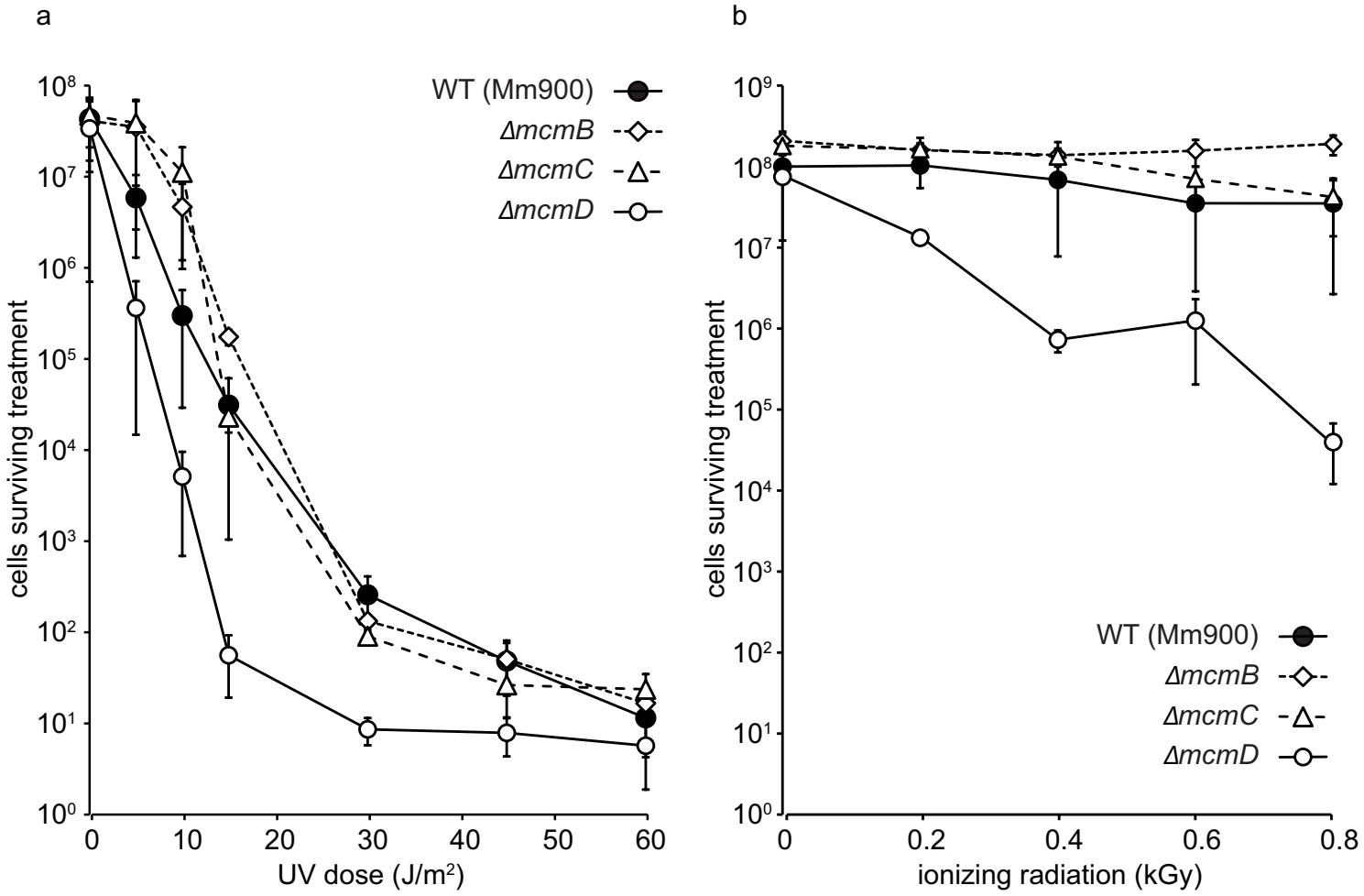
Click here to download Figure Walters_Chong_Figure_4.eps



Walters and Chong - Figure 4

Figure 5

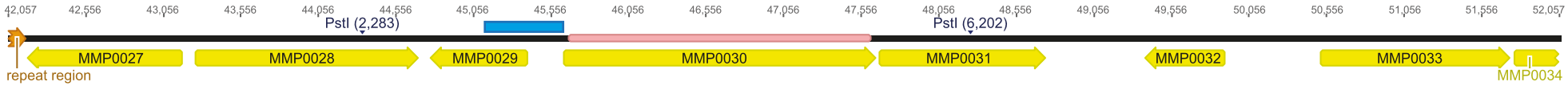
Click here to download Figure Walters_Chong_Figure_5.eps



Walters and Chong - Figure 5

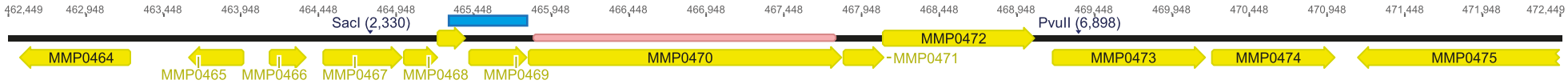
Click here to download Supplementary Material Files Walters_Chong_Supplementary.pdf

McmA



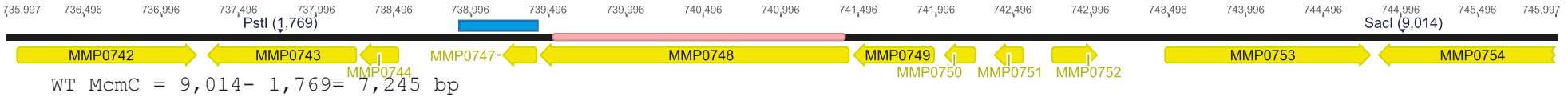
WT McmA = 6,202 - 2,283 = 3,919 bp
mcmA deletion = 2,000 bp

McmB



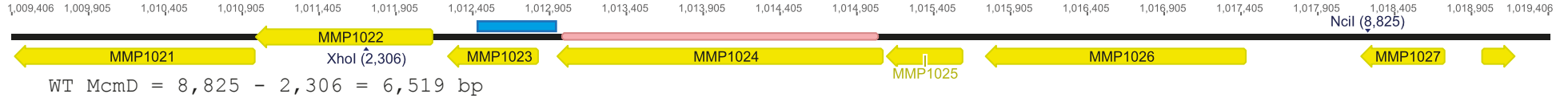
WT McmB = 6,898 - 2,330= 4,568 bp
mcmB deletion = 2,539 bp

McmC



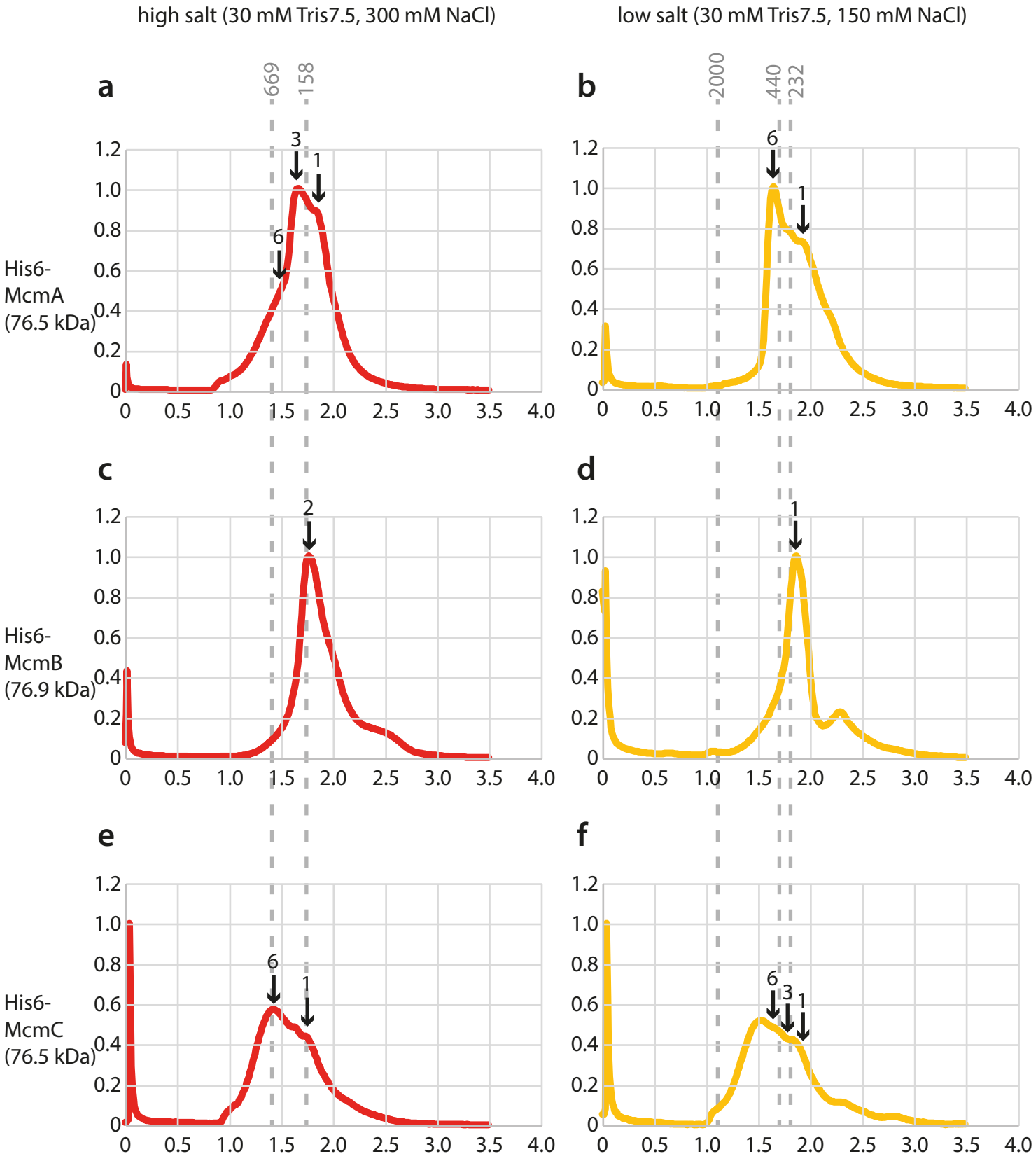
WT McmC = 9,014- 1,769= 7,245 bp
mcmC deletion = 5,245 bp

McmD

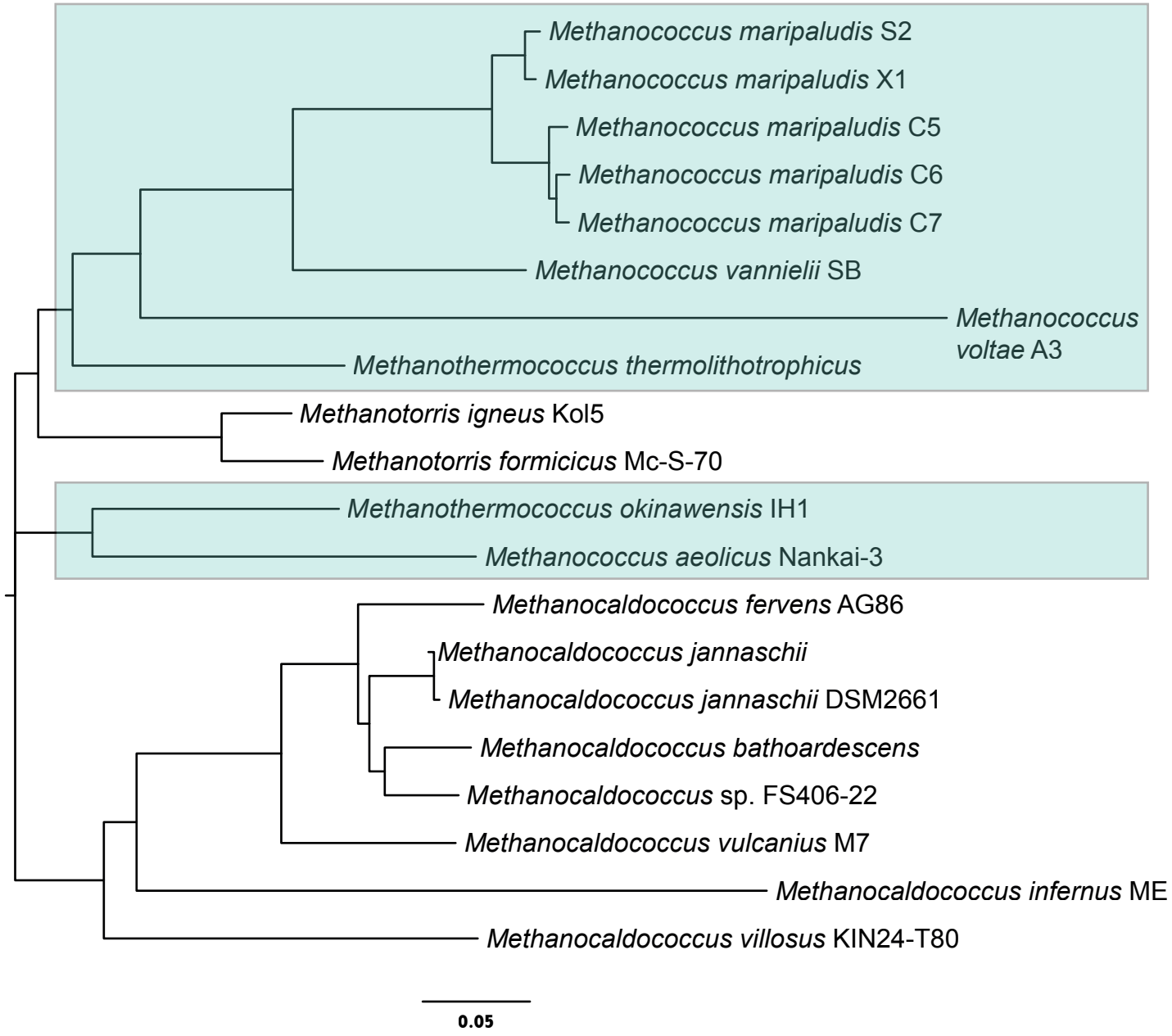


WT McmD = 8,825 - 2,306 = 6,519 bp
mcmD deletion = 4,428 bp

Walters and Chong
Fig. S1
Genomic context for MCM genes with position of restriction enzyme sites used to generate fragments for Southern blots (see Fig. 3). Genomic position in bp indicated at the top of each panel, RE site indicated in bp from beginning of excerpt. Deleted region indicated in red. Probe for Southern blot indicated in blue. Yellow arrows indicate genes and direction of ORF. Text indicates expected fragment sizes for WT and deleted Southen blot fragments.



Walters and Chong
Fig. S2
Absorbance traces (280 nm) from size exclusion chromatography: protein samples were loaded on a 2.6 mL Superose 6 column and eluted at 50 µL/min in the buffer indicated



Walters and Chong
Fig. S3
Phylogenetic tree showing relatedness of all MMP1025 homologues described to date. Boxed genes are found immediately upstream of genes encoding MCM homologues and are likely operonic. MMP1025 homologues are found in all Methanococcales species sequenced to date, correlating with the presence of McmD homologues, but are found in no other species.

```
CLUSTAL 2.1 multiple sequence alignment

M. maripaludis S2          o  ---MDVYDILFLKCTEYEVVVNERHVPLWMLSKSDEERIN--FDLPWTNLQDLAISLYELKREQQKSKELLKCNLEEIIVGISYLKSKKSGSLLSDESMA
M. maripaludis X1          o  ---MDVYDILFLKCTEYEVAVNEKHVPLWMLSKSDEERIN--FDLPWTNLQDLAISLYELKREQQKSKELLKCNLEEIIVGISYLKSKKSGSLLSDESMA
M. maripaludis C6          o  ---MDVYDILFLKCTEYEVVVNERHVPLWMLTEGDEERIN--FDLPWTNLQDLAIYLYELKREQQKSKELLKCNLEEIIVGISYLKSKKSGSLLSDESMA
M. maripaludis C7          o  ---MDVYDILFLKCTEYEVVVNERHVPLWMLTEGDEERIN--FDLPWTNLQDLAIYLYELKREQQKSKELLKCNLEEIIVGISYLKSKKSGSLLSDESMA
M. maripaludis C5          o  ---MDVYDILFLKCTEYEVVVNERHVPLWMLNEGDEERIN--FDLPWTNLQDLAIYLYELKREQQKSKELLKCNLEEIIVGISYLKSKKSGSLLSDESMA
M. vannielii_SB            o  ---MDVYDILFLKCSEYEVLLNEKQIPLWMIKKENALNVN--FDLPWNNLQDLAIYLYELKREQQKSKDLLKCNLEEILVGISYLPSKKSGSLLANESIG
Mcc. jannaschii            x  ---MDVYEILYQFCLEYEVLLDDEKIPLWKLKKEDLDKVD--LDLPWTSIRDLAIYLYELKKKQQNSKELIKCDIVEILVGIALLKPEEGSNYMG--LVT
Mcc. jannaschii DSM2661    x  MKNMDVYEILYQFCLEYEVLLDDEKIPLWKLKKEDLDKVD--LDLPWTSIRDLAIYLYELKKKQQNSKELIKCDIVEILVGIALLKPEEGSNYMG--LVT
Mcc. bathoardescens        x  ---MDVYEILYQFCLEYKVLLNDEEVPLWKLKKDDLEKAN--LDLPWNSIRDLAIYLYELKKKQQNSKELIKCDIVEILVGIALLKPEDGNNYMG--LVT
Mcc. sp. FS406-22          x  ---MDVYETLYQLCLEYKVLLDDKEVPLWKLKKEDLEKAN--LDLPWTSIRDLAIYLYELKKKQQNSKELIKCDIIEILVGIALLKPEEGSNYMG--LVT
Mcc. fervens AG86          x  ---MDVYEILYQSCLEYKVLLNGEETPLWKLKKEDLDKVD--LDLPWTSIRDLAIYLYELKKKQQNSKELIKCDIVEILVGIALLKPEEGNSYMG--LVT
Mcc. vulcanius M7          x  ---MDVYETLYQFCLEYEVLLDDKKVPLWKLKKEDLDSVD--LDLPWNSIRDLAIYLYELKKKQQNSKELVKCDIVEILVGIALLKAEED--YMR--HVH
Mcc. infernus ME           x  ---MDVYETLYNLCLEHEVKVKDKKIPLWKCKS--LEEVED-LNLPWKSLRELTIYLYEVLRTQRESTEFIKFDIVKVLVGLALLREDVYG------VTT
Mcc. villosus KIN24 T80   x  ---MDVYEVLYQACLEYEVVLDGKRVPLWKVKKEDLEKVD--FRLPWNSLRELAVHLYELKSKQQKSKELIRVNLVEILIGIAFLKVEDEFGSIC--NV-
Mtc. okinawensis IH1       o  ---MDVYEVLFQKCLEYEVIVDGKEVPLWKLKKEDIANGNVDFDLQWDSLQDLAISLYELKKEQQKSKELIKYPLEEVIIGIAFLKSKKSGYLITDDMNN
M. aeolicus Nankai-3       o  ---MDVYEVLFQKCLEYEVIIDGKEIPLWKLKKENLDNANFNVNVQWDSLQDLAISIYELKKEQQNSKELIKFPIEEILVAMAFLKSKTKGYLITDDINN
Mt. igneus Kol5            x  ---MDVYEILFQKCLEYEVLLDDEKIPLWKLKKEDLDKVN--FGLPWENLQDLAIYLYELKKEQQRSKELIKCDIAEILVGIAFLKPKKSGSLIADESLG
Mt. formicicus Mc-S-70     x  ---MDVYEILFRKCLEYEVLLDDEKVPLWKLKKEDIDKVN--FGLPWENLQDLAIYLYELKKEQQRSKELIKCDISEILVGIAFLKSEKSNSLIADETLG
Mtc. thermolithotrophicus  o  ---MDVYEVLFEKCLEYEVLLNEKKIPLWKLKKEDLDNVD--FDLPWEHIQDLAIYLYELKREQQKSKELLKCDIDEILVGMAFLKSKKSGSLISDELTG
M. voltae A3               o  ---MDAYSLLFLKCTEYEVYKGETKVPLWQITKEDIKAKNVNFDLPWSSIQDLAITLFDILKDQRRNPDLTYLNLEEILVGISFLNSESSGTLISNQDMA
                                  **.*. *:  * *::*     . ***  ..      :  . : *  :::*:: ::::   *:.. ::    : ::::.:: *  .

M. maripaludis S2          o  IKACMDYLSEFITARINCIYRYYYPMKTPPNKSLFDEVILKFPQKKDIKAKNRQDFEEIISKLKKYDFNLQN---
M. maripaludis X1          o  IKACMDYLSEFITARINCIYRYYYPMKTPPNKSLFDEVILKFPQKKDIKAKNRQDFEEIISKLKKYDFNLQN---
M. maripaludis C6          o  IKACMDYLSEFITARINCIYRYHYPMKTPANKSLFDEVILKFPQKKDIKAKNRQDFEEVISRLKKYNFNLQN---
M. maripaludis C7          o  IKACMDYLSEFITARINCIYRYHYPMKTPANKSLFDEVILKFPQKKDIKAKNRQDFEEVISRLKKYDFTLQN---
M. maripaludis C5          o  IKACMDYLSEFITARINCIYRYHYPMKTPTNKSLFDEVILKFPQKKDIKAKNRQDFEEVISRLKKYDFNLQN---
M. vannielii_SB            o  IDACLSYLSEFITARINCIYRYHYPMTVPVNKSLFDEVILKFPQKKDVKAKNKHDFEYIVSKLKNYDFKLQFKRN
Mcc. jannaschii            x  EDMCLTYLSELITARINCIARYYYMMKKPQNTNIFDEIILKFPQKKDIRASNINDLRELVGKIRNY-FK------
Mcc. jannaschii DSM2661    x  EDMCLTYLSELITARINCIARYYYMMKKPQNTNIFDEIILKFPQKKDIRASNINDLRELVGKIRNY-FK------
Mcc. bathoardescens        x  EDMCLTYLSELITARINCIARYYYMMKKPQNTNIFDEIILKFPQKKDIRASNINDLRELICKIRNY-FK------
Mcc. sp. FS406-22          x  EDMCLTYLSELITARINCIARYYYMMKKPQNTNIFDEIILKFPQKKDIRASNINDLRDLVGKIRNY-FK------
Mcc. fervens AG86          x  EDMCLNYLSELITARINCIAKYYYMMKKPQNTNIFDEIILKFPQKRDIRASNINDFRELVGRIRSY-FK------
Mcc. vulcanius M7          x  EDTCLRYLSELITARINCIAKYYYMMKKPHNTDIFDEIILKFPQKKDLRASNINDLRLLIDRIRGY-FE------
Mcc. infernus ME           x  EETALKYLSQIITYRMNILARYYYLIKKPINTSIFEDIILKFPQNRDIRTSNIEDLKILVEKIKKR-FKP-----
Mcc. villosus KIN24 T80    x  EDLCLTYLSELITARINCIAKYYYLIKKPNNTDLFDEIILKFPQNKNIKAGNLNDLKELIFKLKTY---------
Mtc. okinawensis IH1       o  INTCLNYLSELITARINCISRYYYLIKKPMNTNLFDEIILKFPQKKDIKVKNIEDLKELVFKLKNF-GKNLKI--
M. aeolicus Nankai-3       o  INTCLSHLSDLITARLNCIFRYYYLMKKPVNTNIFDRVVLKFHQQKNIKVNNLNDFQKIVFKLKNLDFEY-----
Mt. igneus Kol5            x  INTCLNYLSELITARINCITRYYYLMKKPHNTDIFDEIILKFPQKKDIRAKNINDLREIVYKLRSY-FEK-----
Mt. formicicus Mc-S-70     x  INTCLNYLSELITARINCITRYYYLMKKPHNTDIFDEIILKFPQKKDVRAKNINDLKEIVYRLKDY-FE------
Mtc. thermolithotrophicus  o  IKTCLNYLSELITARINCIARYHYLMKNPGNRNIFDDVILKFPQKKDVKVKNTEDLEKIVFKLKNLDFNYD----
M. voltae A3               o  TIACINHLDDLLSTRISKICAHNVLMKMPETACLFEKIAFGFPQKKDVKITVNPELTKIIQRLRNCEFESELLN-
                                  .: :*.:::: *:. :  :   :. * .  :*: : : * *:::::     ::  :: :::
```

Walters and Chong, Fig. S4: ClustalX alignment of all existing MMP1025 homologues. ‘o’ indicates genes that are upstream and likely operonic with McmD homologues, ‘x’ indicates non-operonic genes.