An archaeal order with multiple minichromosome maintenance genes

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In eukaryotes, a complex of six highly related minichromosome maintenance (MCM) proteins is believed to function as the replicative helicase. Until recently, systems for exploring the molecular mechanisms underlying eukaryotic MCM function have been biochemically intractable. To overcome this, molecular studies of MCM function have been carried out using MCM homologues from the archaea. Archaeal MCM systems studied to date possess a single functional MCM, which forms a homohexameric complex that displays DNA binding, ATPase and helicase activities. We have identified an archaeal order that possesses multiple MCM homologues. BLAST searches of available Methanococcales genomes reveal that members of this order possess between two and eight MCM homologues. Phylogenetic analysis suggests that an ancient duplication in the Methanococcales gave rise to two major groups of MCMs. One group contains *Methanococcus maripaludis* S2 McmD and possesses a conserved C-terminal insert similar to one observed in eukaryotic MCM3, while the other group contains McmA, -B and -C. Analysis of the genome context of MCMs in the latter group indicates that these genes could have arisen from phage-mediated events. When co-expressed in *Escherichia coli*, the four MCMs from *M. maripaludis* co-purify, indicating the formation of heteromeric complexes *in vitro*. The presence of homologues from both groups in all Methanococcales indicates that there could be functionally important differences between these proteins and that Methanococcales MCMs may therefore provide an interesting additional model for eukaryotic MCM function.

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INTRODUCTION

The archaea make up a third domain of life that is best known for including a large number of 'extremophilic' organisms. This group of prokaryotes was first identified as being distinct from bacteria by differences in their 16S rRNA sequences (Woese & Fox, 1977). Methanogens were the first organisms to be identified as archaea and classified as a separate domain (Woese & Fox, 1977). Five orders of methanogens exist: Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales and Methanopyrales (Thauer, 1998). Complete genome sequences for representatives of all of these orders are available. Initial analyses of the *Methanocaldococcus* (previously *Methanococcus*) *jannaschii* genome have revealed that the archaea have many metabolic characteristics in common with bacteria, but that the genes used for information processing

are more similar to equivalent systems in eukaryotes (Bult *et al.*, 1996; Edgell & Doolittle, 1997). The observation that archaeal information-processing systems are similar to but simpler than those in eukaryotes, along with the thermostable nature of proteins from many of these organisms, has led to a number of archaeal species being used as models for a variety of eukaryotic processes (Tye, 2000).

DNA replication is a good example of how a eukaryotic process is simplified in archaea. The heterohexameric minichromosome maintenance (MCM) complex in eukaryotes is believed to be the genomic DNA helicase; that is, the protein that is responsible for unwinding genomic DNA prior to replication (Labib *et al.*, 2000, 2001; Prokhorova & Blow, 2000). A range of different MCM complexes have been isolated from eukaryotic systems. The heteromeric nature of these complexes and the requirement for all six proteins has posed a significant hurdle to a comprehensive biochemical characterization of the MCM helicase. In archaea that have been characterized to date, a single MCM protein forms a homohexameric complex that possesses ATP-dependent DNA helicase activity. MCM complexes from a number of species (most notably *Sulfolobus* and *Methanothermobacter*) have been characterized and have provided insight into the mechanisms that govern helicase activity in these proteins. Motifs essential for

Abbreviations: GST, glutathione S-transferase; MCM, minichromosome maintenance; ML, maximum-likelihood; NJ, neighbour-joining.

A supplementary table, showing the results of a codon-based comparison of synonymous changes in MCM sequences from the same species, and two supplementary figures, showing an alignment of eukaryotic MCMs, and the genome context of McmA and McmD genes in the Methanococcales, are available with the online version of this paper.

MCM function have been identified using ATP hydrolysis, DNA binding and DNA helicase activity assays (Barry *et al.*, 2007; Carpentieri *et al.*, 2002; Chong *et al.*, 2000; Jenkinson & Chong, 2006; Kasiviswanathan *et al.*, 2004; Kelman *et al.*, 1999; Poplawski *et al.*, 2001; Shechter *et al.*, 2000).

The genome sequence of *Methanocaldococcus jannaschii* has revealed that in contrast to the single MCM found in other archaea, this organism has multiple, apparently functional MCM homologues (Bult *et al.*, 1996). Analysis of the genome sequence of *Methanococcus maripaludis* S2 has revealed that it also contains multiple MCMs (Hendrickson *et al.*, 2004; Walters & Chong, 2009). The four *M. maripaludis* S2 MCM homologues are distributed across the genome and are assigned ORF numbers that we have further supplemented with gene names (indicated in parentheses) as MMP0030 (*mcmA*), MMP0470 (*mcmB*), MMP0748 (*mcmC*) and MMP1024 (*mcmD*). Sequence analysis of additional Methanococcales genomes has shown that all of them possess multiple MCM homologues. Our results suggest that the MCM genes underwent an ancient duplication in the order Methanococcales, probably before the current species evolved. In addition, we present evidence that two distinct classes of MCM exist in the Methanococcales, one of which has been duplicated through the action of a mobile element that can still be observed on a cryptic plasmid found in these species; the other has similarities to eukaryotic MCM3. Co-expression of recombinant MCMs from *M. maripaludis* S2 allowed co-purification of all four proteins, indicating that they form one or more heteromeric complexes. Our data support the hypothesis that the Methanococcales are unique among the archaea in possessing multiple functional MCMs and may therefore be a good model for understanding eukaryotic MCM interactions.

METHODS

MCM identification. To identify MCM homologues, the MMP0030 (*McmA*) protein was used as the query sequence in a BLASTP (Altschul

et al., 1997) search against the NCBI non-redundant database (Pruitt *et al.*, 2007) with a cut off value of $1e^{-20}$.

Phylogenetic analyses. A multiple sequence alignment containing 31 potentially functional Methanococcales MCM homologues (listed in Table 1) plus an outgroup of Methanobacteria (*Methanothermobacter thermautotrophicus*, *Methanobacterium stadtmanae* and *Methanobrevibacter smithii*) and *Methanopyrus kandleri* was constructed in CLUSTAL_X (Thompson *et al.*, 1997). The alignment of 35 sequences was edited using Gblocks (Castresana, 2000). This edited alignment was used to generate a phylogenetic tree using both neighbour-joining (NJ) and maximum-likelihood (ML) methods. ML phylogenetic analyses were performed using PHYML (Guindon & Gascuel, 2003) with the JTT substitution model applied (Jones *et al.*, 1992). One hundred bootstrap replicates were generated for ML analysis and 1000 replicates for the NJ analysis. The two different methods gave the same basic tree topology.

Identification of homologous flanking genes. Related gene contexts of MCMs were initially identified using genome comparison tools at <http://theseed.uchicago.edu/FIG/index.cgi> (Overbeek *et al.*, 2005).

Assembly of contextual maps. The contextual arrangement of genes surrounding Methanococcales MCM homologues was visualized using Lasergene (Burland, 2000). Homologous genes were identified in other genomes from the order using BLASTP (Altschul *et al.*, 1997), using a cut-off of $1e^{-20}$.

Identification of repeat sequences. DNA sequences in the region of MCM genes were examined for repeat sequences using MEME (Bailey & Elkan, 1994) with the following parameters: any number of repetitions, minimum width 6, maximum width 50, up to 5 motifs found.

Expression and purification of *M. maripaludis* S2 MCMs. The four *M. maripaludis* S2 MCMs were amplified by PCR from genomic DNA. *mcmA* and *mcmC* were cloned into pCDF-2 Ek/LIC (Novagen) with a T7-tag adaptor (Novagen) using ligation-independent cloning such that *mcmA* had an N-terminal hexa-histidine tag and *mcmC* had an N-terminal T7 tag. *mcmB* and *mcmD* were cloned into pET32 Ek-LIC (Novagen) with a glutathione S-transferase (GST) adaptor such that *mcmB* had an N-terminal Trx.hexa-histidine.S tag and *mcmD* had an N-terminal GST tag. The expression constructs were co-transformed into Rosetta BL21(DE3) *Escherichia coli* cells (Novagen). Cells were grown with shaking (200 r.p.m.) at 37 °C until the OD₆₀₀ reached 0.8

Table 1. Methanococcales MCM homologues identified by BLASTP searches using MMP0030 (*McmA*) as the query sequence

The genome sizes of all organisms are similar, but the number of MCMs ranges from two to eight. *Methanocaldococcus jannaschii* and *M. vannielii* both possess truncated MCM homologues that lack motifs essential for function (ORF numbers for truncated MCMs are shown in parentheses).

Organism	Genome size (Mb)	Total MCM homologues	Number of potentially functional MCMs	MCM ORF numbers
<i>M. maripaludis</i> S2	1.66	4	4	MMP0030; MMP0470; MMP0748; MMP1024
<i>M. maripaludis</i> C5	1.81	4	4	MmarC5_0570; MmarC5_0643; MmarC5_1119; MmarC5_1650
<i>M. maripaludis</i> C6	1.7	8	8	MmarC6_0030; MmarC6_0108; MmarC6_0133; MmarC6_0420; MmarC6_0438; MmarC6_0919; MmarC6_1635; MmarC6_1837
<i>M. maripaludis</i> C7	1.8	4	4	MmarC7_0027; MmarC7_0065; MmarC7_0266; MmarC7_1027
<i>M. aeolicus</i> Nankai-3	1.6	3	3	Maeo_0490; Maeo_1144; Maeo_1226
<i>M. vannielii</i> SB	1.7	4	3	Mevan_0267; Mevan_0348; Mevan_1027 (Mevan_1640)
<i>M. voltae</i> A3	1.86	2	2	MvolDRAFT_0034; MvolDRAFT_0575
<i>Methanocaldococcus jannaschii</i>	1.76	4	3	Mjan_0363; Mjan_0961; Mjan_1489 (Mjan_ECL13)

and then chilled on ice for 20 min before induction with 0.1 mM IPTG. Cultures were incubated for a further 24 h at 12 °C with 200 r.p.m. shaking before cells were harvested. Cells were resuspended in 2 ml g⁻¹ lysis buffer (25 mM Tris, pH 8.0, 150 mM NaCl, 10 %, v/v, glycerol, 1 mM PMSF, 1 µg leupeptin ml⁻¹, 1 µg pepstatin ml⁻¹, 1 µg aprotinin ml⁻¹), supplemented with 0.75 mg lysozyme ml⁻¹ and 5 µg DNase ml⁻¹. Cells were lysed by sonication and the lysate was clarified by centrifugation at 50 000 g for 30 min.

McmD and associated proteins were purified from 1.5 l culture using 250 µl glutathione–Sephacel beads (GE Healthcare). After the addition of cell lysate, beads were washed with 10 column volumes of lysis buffer (as above) and eluted in 50 mM Tris, pH 8.0, 150 mM NaCl, 40 mM reduced glutathione. For the multi-step purification, MCM protein was purified from 4.5 l culture using nickel–Sephacel beads (GE Healthcare), eluted in 25 mM Tris, pH 8.0, 150 mM NaCl, 10 %, v/v, glycerol, 500 mM imidazole. Elution fractions totalling 2 ml from the nickel–Sephacel purification were pooled and further purified by size-exclusion chromatography using a Superdex 200 16/60 (GE Healthcare) column in 25 mM Tris, pH 8.0, 150 mM NaCl, 10 %, v/v, glycerol at a flow rate of 1 ml min⁻¹. Fractions containing MCMs were identified by SDS-PAGE, pooled and immediately loaded onto a 1 ml Source Q column (GE Healthcare), then eluted in 25 mM Tris, pH 8.0, 10 %, v/v, glycerol, 1 mM PMSF, 1 µg leupeptin ml⁻¹, 1 µg pepstatin ml⁻¹, 1 µg aprotinin ml⁻¹ over a 30 ml 50–1000 mM NaCl gradient at a flow rate of 1 ml min⁻¹. Elution fractions were separated by SDS-PAGE and analysed by Western blotting using anti-His (McmA), anti-S (McmB), anti-T7 (McmC) and anti-GST (McmD) antibodies.

RESULTS

Identification of MCM homologues in Methanococcales

Complete genome sequences exist for *M. maripaludis* strains S2, C5, C6 and C7, *Methanocaldococcus jannaschii*, *Methanococcus aeolicus* Nankai-3 and *Methanococcus vannielii* SB (<http://www.ncbi.nlm.nih.gov/sites/genome/>). Most of the *Methanococcus voltae* A3 genome is also complete, although a single contiguous sequence is not available. BLAST searches were carried out against each of the eight Methanococcales genomes using McmA from *M. maripaludis* S2 as the query sequence. Hits with an expect score of 1e⁻²⁰ or better were considered to be reliable MCM homologues. Results showed that all of the Methanococcales genomes contain multiple MCMs. MCM homologue numbers range from two (in *M. voltae* A3) to eight (in *M. maripaludis* C6) (Table 1). Genome sizes are similar in all eight organisms, indicating that there have been no recent large genome duplications that have led to, for example, the doubling of MCM number in *M. maripaludis* C6 compared with *M. maripaludis* S2.

Multiple sequence alignments of Methanococcales MCMs

Multiple sequence alignments were generated from all the MCM homologues identified in *Methanococcus* genomes along with MCM homologues from three Methanobacteria. The alignment shows that of the 33 Methanococcales MCM sequences, 30 contain the known motifs required for MCM function (Fig. 1). Of those not possessing all the

required motifs, two are truncated and do not contain the arginine finger required for ATP hydrolysis. One of these truncated proteins is found on an extra-chromosomal element in *Methanocaldococcus jannaschii* (MJ_ECL13) and the other is on the main chromosome of *M. vannielii* (Mevan_1640). The third protein showing significant changes in the normally conserved MCM motifs was ORF 0961 of *Methanocaldococcus jannaschii*. This protein contains a large insertion between the first two cysteines of the zinc finger, and lacks the second pair of cysteines.

Interestingly, a highly conserved 20 amino acid insertion is found in one MCM homologue from each of the eight Methanococcales analysed. This insertion is not present in any other archaeal MCMs and is particularly interesting because analysis of the six eukaryotic MCM sequences has shown that an insertion of similar size and in an equivalent position is unique to MCM3 (Supplementary Fig. S1). Evidence suggests that MCM3 may play a regulatory role in the eukaryotic MCM complex (Lin *et al.*, 2008; Shi *et al.*, 2007; Takei *et al.*, 2002), so it is possible that this insertion plays a role in regulating MCM activity in the Methanococcales. The C-terminal domain of other archaeal MCMs has been shown to regulate MCM activity *in vitro* (Grainge *et al.*, 2003; Jenkinson & Chong, 2006), and therefore any insertions in the C terminus could potentially alter its regulatory activity. The fact that there is an MCM homologue with a similar insertion in each of the eight Methanococcales genomes suggests that this protein plays a role different from the other Methanococcales MCMs, and indeed MCMs found in other archaea.

Phylogenetic analysis of Methanococcales MCMs

In order to gain some insight into the evolution of MCMs in the Methanococcales, phylogenetic analysis was carried out on all of the full-length Methanococcales MCM sequences available (Fig. 2). The tree shows that the Methanococcales MCMs are more closely related to each other than they are to members of the outgroup, which is representative of other methanogen MCMs. The tree also shows that there have been multiple duplications of MCMs within the Methanococcales. An ancient duplication that pre-dates the divergence of the Methanococcales into separate species appears to have given rise to two major groups of MCMs. One of these groups contains one MCM homologue from each of the eight Methanococcales species analysed. This group contains McmD from *M. maripaludis* S2 and has therefore been named the 'McmD' group (Fig. 2). All eight members of this group contain the conserved 20 amino acid C-terminal insert identified above. The McmD group is strongly supported by high bootstrap values for both NJ and ML analyses. The second group produced from the ancient duplication has undergone further duplications to generate several subgroups of MCMs. One of these subgroups includes a single homologue from each of the eight Methanococcales species analysed. This subset of eight proteins includes McmA from *M. maripaludis* S2 and has therefore been named the 'McmA' group. There are three subgroups related to the McmA group

Fig. 1. Summary of observations from a multiple sequence alignment of Methanococcales MCMs. Conserved motifs important for function are represented by boxes containing consensus sequences. H2i, helix-2 insert; PS1BH, pre-sensor 1 β -hairpin; R-finger, arginine finger. Alignments for each conserved motif are shown in full. A 20 amino acid insert is conserved at the C terminus of one MCM homologue in all organisms analysed, represented by McmD in *M. maripaludis* S2. MCMs from *M. maripaludis* C5, C6 and C7 are not included in the expanded alignments, but contain the same conserved motifs as *M. maripaludis* S2.

Fig. 2. ML phylogeny of the Methanococcales MCMs. ML and NJ trees were drawn using the same 272 amino acid alignment. A total of 1000 bootstraps were performed for the NJ tree and 100 bootstraps for the ML tree. Percentage bootstrap values are shown for the major groups recovered using ML and NJ (indicated in parentheses) analyses. Only bootstrap values over 75 % are shown. The McmA and McmD groups are highlighted by shaded boxes. Other Methanococcales groups are labelled 1–4. MMP, *M. maripaludis*; Msm, *Methanobrevibacter smithii*; Mstad, *Methanobacterium stadtmannae*; Mth, *Methanothermobacter thermophilus*; Mkan, *Methanopyrus kandleri*; Mjan, *Methanocaldococcus jannaschii*; Maeo, *Methanococcus aeolicus*; Mvol, *M. voltae*; Mvan, *M. vannielii*.

(Fig. 2, groups 2, 3 and 4) that have been produced by a number of further duplications, but these duplications are not common to every species.

As an additional measure of whether the multiple MCM genes in each Methanococcales species were likely to retain function, we measured selective pressure on the genes through the pairwise comparison of dS/dN ratios of genes in each species. Our codon-based analysis indicated that within all individual species synonymous mutations were favoured over non-synonymous changes. Together with the conservation of all known motifs required for MCM activity, this result is consistent with the notion that all MCM homologues could be functional (Supplementary Table S1).

Genome context analysis of *Methanococcales* MCMs

The phylogenetic tree (Fig. 2) shows that there have been several duplications of MCMs within the Methanococcales. To investigate the nature of these duplications, the genome

context of all full-length Methanococcales MCMs was compared (Fig. 3 and Supplementary Fig. S2). The comparison showed that all of the MCMs outside the McmA and McmD groups (Fig. 2) are found close to an integrase homologue (Fig. 3, yellow). There is an 83 amino acid ORF that is present upstream of the integrase gene in several MCM regions that is also found in the cryptic plasmid pURB500, which was first isolated from *M. maripaludis* C5 (Tumbula *et al.*, 1997). In most cases, the integrase homologue is annotated as a phage integrase (Groth & Calos, 2004), and is found at varying distances from MCM ORFs. In some cases, such as in the region of MmarC5_1119 (Fig. 3, group 4), the integrase is annotated as a pseudogene; however, a BLASTX search with the nucleotide sequence from this ORF showed that it is a phage integrase homologue ($E=6e^{-171}$). Genes found in the McmA and McmD groups are not associated with an integrase (Supplementary Fig. S2). A closer examination of the genome context of MCMs falling outside the McmA and McmD groups revealed that MCMs that cluster into separate groups in the phylogenetic analysis (groups 1–4,

Fig. 3. Genome context analysis of Methanococcales MCMs. Analyses suggest that MCMs that fall outside the *mcmA* and *mcmD* groups have been generated by duplications involving a phage or mobile element. Groups 1–4 are numbered to correspond with the groups identified in Fig. 2. A phage integrase homologue (yellow) is located close to each MCM (red). MCMs that cluster into separate groups by phylogenetic analysis contain different sets of homologous flanking ORFs, suggesting that several different integration events involving MCMs have occurred. Homologous ORFs within each group are colour coded. ORFs coloured grey show no homology to others in the group. Group 1 MCMs have only two common flanking genes. Group 2 MCMs form two distinct pairs and each is associated with a tRNA gene (vertical black triangles), which is a common site for integration events (Campbell, 1992). Restriction-modification system genes (boxed) are present close to MCMs in group 2 and genes encoding phage structural proteins are present in group 4 (black), providing further evidence for the role of phages in these MCM duplications. Sizes of DNA regions are indicated above each group in base pairs. ORF numbers for MCM and integrase genes are also indicated. Species names are abbreviated as described in the legend to Fig. 2.

Fig. 2) contain different sets of homologous flanking ORFs (Fig. 3), suggesting that several separate integration events involving MCMs have occurred. The presence of homologous flanking genes between groups 3 and 4, and their close phylogenetic relationship indicate that they could have been produced by similar integration events, or by a single integration event followed by a duplication.

Expression and co-purification of *M. maripaludis* S2 MCMs

The six eukaryotic MCMs form a heterohexameric complex, which is believed to contain one of each of the MCM2–7 proteins (Labib *et al.*, 2000, 2001; Prokhorova & Blow, 2000). In order to identify whether the multiple MCMs from *M. maripaludis* S2 could also form a

heteromeric complex, the four MCM proteins were co-expressed then purified from *E. coli*. Constructs were generated that allowed the co-expression of all four MCMs, each with a different tag at the N terminus (His-McmA, TrxHisS-McmB, T7-McmC, GST-McmD). Protein was initially purified by affinity chromatography using glutathione-Sepharose beads, to which GST-tagged McmD should bind specifically. Western blot analysis of the resulting elution fractions using antibodies specific to each of the tagged MCMs showed that all four MCMs are present in elution fractions 1 and 2 (Fig. 4a). The differences between the levels of signal for each protein seen in the Western blots (Fig. 4) are most likely due to differing antibody specificities, although expression levels and abundance of individual proteins in the isolated complexes may also account for the apparent variation. In order to validate the co-purification of the four *M. maripaludis* S2 MCMs in the single-step GST purification, a multi-step purification was carried out. Protein was purified from cell extract co-expressing all four MCMs first by affinity chromatography using nickel-Sepharose beads, then by size-exclusion chromatography and finally by anion exchange. Western blot analysis of elution fractions from the third purification step showed that all four MCMs were present in the peak anion-exchange fraction 3 (Fig. 4b). The co-purification of McmA, -B, -C and -D through multiple purification steps indicates that the four MCMs form one or more heteromeric complexes *in vitro*. However, the stoichiometry of the heteromeric complex(es) formed cannot be calculated from these experi-

ments, and it is possible that several heteromeric complexes composed of different MCM subunits could be formed.

DISCUSSION

The Methanococcales are unique among the archaea in possessing multiple apparently functional MCM genes. While a few other archaeal genomes encode more than one MCM sequence, in all cases only one MCM gene possesses all the motifs known to be required for activity (Galagan *et al.*, 2002; McGeoch & Bell, 2008; Slesarev *et al.*, 2002). The number of MCMs in the Methanococcales ranges from two in *M. voltae* A3 to eight in *M. maripaludis* C6. The ML and NJ phylogenies that we have produced are consistent with there being at least two MCM homologues that play important functional roles in the Methanococcales. These two homologues are present in all eight species analysed and appear to have been created by an ancient duplication that pre-dates the divergence of the different Methanococcales species. MCMs that do not fall directly within the McmA or McmD groups are all associated with an integrase homologue, suggesting that duplication of these genes is due to the action of a mobile element.

Mechanisms of MCM duplication

A number of mechanisms for the duplication of integrase-associated MCMs are possible and include (i) the activity of insertion sequence (IS) elements, (ii) transposon activity and (iii) phage-mediated duplication. Although IS ele-

Fig. 4. All four *M. maripaludis* MCM proteins co-purify when co-expressed in *E. coli*. (a) Cells induced to co-express all four *M. maripaludis* S2 MCMs were harvested and lysed to produce a cell-free extract (soluble and insoluble fractions are shown). The soluble fraction was passed over a GST column in order to affinity-purify GST-McmD. The column was extensively washed before 250 μ l fractions were eluted using reduced glutathione (E1–E5). Western blots using antibodies specific to each of the differently tagged MCMs (as indicated) showed that all four recombinant MCMs co-purify in this single-step affinity purification. Variability in detection is likely due to varying sensitivity of different antibodies. (b) A purification similar to that above was carried out using a multi-step protocol using nickel-affinity (Ni^{2+}) chromatography, size-exclusion chromatography and anion-exchange columns. Samples from 0.5 ml elution fractions from the final column are shown (1–5). Co-elution of all four proteins was detected in fraction 3.

ments have been identified in *Methanocaldococcus jannaschii* (Bult *et al.*, 1996), we have been unable to identify any obvious IS elements in the regions where the duplications have occurred. Likewise, searches for repeated sequences characteristic of transposon-type activity have not yielded any noteworthy results. However, the presence of a phage integrase and a smaller associated ORF in the same region as every MCM outside of the McmA and McmD groups is consistent with a phage-mediated duplication of MCMs in the Methanococcales. tRNA sequences, a common site of phage integration in prokaryotes (Reiter *et al.*, 1989), are present in the same region as a number of integrase-associated MCMs (Fig. 3, group 2), supporting the hypothesis that this duplication was phage-mediated. The presence of putative phage structural proteins (Fig. 3, group 4) and restriction-modification system genes (Fig. 3, group 2) close to some integrase-associated MCMs is further evidence for the involvement of phages in these MCM duplications. To date, there have been no phages described for the Methanococcales, so there are no obvious candidate phages for involvement in these MCM duplications. However, an association between phage and MCM proteins has been observed in the *Bacillus cereus* genome, where an MCM homologue is located in an integrated bacteriophage (McGeoch & Bell, 2005).

Functions of multiple MCMs

A number of other methanogenic archaea also possess more than one MCM sequence, but in these cases only a single MCM has all of the motifs required for MCM function, suggesting that a single MCM is sufficient for DNA replication and that MCM function in these other duplications has been lost (Bae *et al.*, 2009; McGeoch & Bell, 2008). Why the Methanococcales are the only order of archaea in which MCM sequences have been duplicated and conserved is not clear. It is possible that only a single MCM is functional, as in other archaea, and that the additional homologues are inactive and not required. However, a shotgun proteomics study carried out in *M. maripaludis* S2 has detected peptides for McmA, -B and -D, indicating that multiple MCMs are expressed (Xia *et al.*, 2006). No peptides were detected for McmC, but this does not exclude the possibility of McmC expression, as peptides may have been present but not detected, or this protein may be expressed only under specific circumstances. Our bioinformatic analysis together with the proteomics data from Xia and colleagues strongly suggest that multiple MCMs are functional in *M. maripaludis*.

The fact that the MCMs in the Methanococcales have retained the motifs for function suggests either that the duplications have occurred very recently and there has not been sufficient time to accumulate mutations, or that the presence of multiple functional MCMs is in some way advantageous to the cells. Phylogenetic analyses do not support the notion that MCM duplications occurred

recently, implying that multiple potentially functional MCM homologues have been retained because they benefit the cell. A codon-based examination of the rates of synonymous versus non-synonymous changes in MCM sequences within a species suggests that a positive selection pressure exists which may be responsible for retaining function in these proteins. In the case of integrase-associated MCMs, it is possible that the advantage in retaining these functions may not be directly cellular, but related to the mobile-element activity associated with these regions. That is, mobile elements are replicated independently of the host genome and require a functional MCM protein to replicate and/or to integrate into the genome. It is also possible that a plasmid-encoded MCM has been integrated into the genome and has been conserved because it is required for plasmid maintenance. Alternatively, multiple MCMs may have been conserved because they provide functions that are beneficial to chromosomal DNA replication. This seems most likely in the case of McmA and McmD homologues, which are not integrase-associated and are conserved in all Methanococcales species analysed. While there could be some redundancy among the different MCM homologues, it is also possible that some have diverged to perform specialized functions. In eukaryotes, a degree of specialization has occurred with MCM duplication. The eukaryotic proteins appear to have subtly different roles in the regulation of helicase loading and unwinding (Sheu & Stillman, 2006; Tsuji *et al.*, 2006), and potentially other functions such as triggering DNA damage checkpoints (Cortez *et al.*, 2004; Tsao *et al.*, 2004). The co-purification of all four recombinant MCMs from *M. maripaludis* S2 (Fig. 4) indicates that these proteins could form heteromeric complexes with different subunits potentially performing specialized roles.

Both the bioinformatic and *in vitro* co-purification data that we have produced indicate that the Methanococcales possess a unique complement of MCM proteins that warrants further genetic and biochemical characterization. The multiple potentially functional MCMs in the Methanococcales could provide a model system intermediate between the heteromeric eukaryotic MCM system and the current archaeal systems, where only one MCM is present, for investigating the molecular mechanisms that underlie the enzymic and regulatory roles of different subunits in systems possessing multiple MCMs.

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