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# Stimulation of MCM helicase activity by a Cdc6 protein in the archaeon *Thermoplasma acidophilum*

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## ABSTRACT

**Replicative DNA helicases are ring-shaped hexamers that play an essential role in chromosomal DNA replication. They unwind the two strands of the duplex DNA and provide the single-stranded (ss) DNA substrate for the polymerase. The minichromosome maintenance (MCM) proteins are thought to function as the replicative helicases in eukarya and archaea. The proteins of only a few archaeal organisms have been studied and revealed that although all have similar amino acid sequences and overall structures they differ in their biochemical properties. In this report the biochemical properties of the MCM protein from the archaeon *Thermoplasma acidophilum* is described. The enzyme has weak helicase activity on a substrate containing only a 3′-ssDNA overhang region and the protein requires a forked DNA structure for efficient helicase activity. It was also found that the helicase activity is stimulated by one of the two *T.acidophilum* Cdc6 homologues. This is an interesting observation as it is in sharp contrast to observations made with MCM and Cdc6 homologues from other archaea in which the helicase activity is inhibited when bound to Cdc6.**

## INTRODUCTION

DNA replication is a key event for cell proliferation and requires the coordinated activity of multiprotein complexes. The process can be divided into three main phases: initiation, elongation and termination. Much of the regulation takes place at the initiation stage, during which the origin of replication is recognized by a protein complex, leading to the assembly of the helicase onto DNA.

To date, only limited information is available regarding the mechanism of initiation in archaea. Primary amino acid sequence analysis suggested that the archaeal genomes contain homologues of the eukaryotic minichromosome maintenance (MCM) helicase and homologues of the eukaryotic origin recognition complex (ORC) and/or the initiator protein Cdc6 (1,2). As the eukaryotic Cdc6 and subunits of ORC show amino acid sequence similarity and the function(s) of the archaeal homologues have not yet been determined, they will be referred to as Cdc6.

The archaeon *Thermoplasma acidophilum* is a thermophilic microorganism from the euryarchaea kingdom. It was isolated from self-heated smoldering coal refuse piles and has an optimal growth temperature of 59°C and a pH of 2 (3). One of the interesting features of the organism is the lack of a cell wall and thus it was originally considered a mycoplasma. Its genome consists of a single circular chromosome of 1.56 Mbp and contains ∼1500 open reading frames (4) with a large number of genes thought to be laterally transferred from *Sulfolobus solfataricus*, a phylogenetically distant crenarchaeon that inhabits similar environments (5).

To date *in vitro* studies on the proteins participating in the initiation of archaeal DNA replication have focused on three organisms: *Methanothermobacter thermautotrophicus*, *S.solfataricus* and *Archaeoglobus fulgidus*. Some *in vivo* studies have been conducted in *S.solfataricus*, *S.acidocaldarius* and *Pyrococcus abyssi*. These studies showed that although the proteins participating in the initiation process are similar in primary amino acid sequence and overall structure, they exhibit different biochemical properties. Thus, in order to further our understanding of the mechanism of DNA replication in archaea and to explore the diversity of the replication machinery in this domain, a study on the replication machinery of *T.acidophilum* was initiated.

It is shown here that the single MCM homologue of *T.acidophilum* and two Cdc6 proteins contain several biochemical properties not yet reported for the enzymes from other archaea. The MCM helicase possesses a very weak helicase activity on a substrate containing only a 3′-single-stranded (ss) DNA overhang region. The activity is profoundly stimulated in the presence of a forked DNA

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substrate (containing both 3′- and 5′-overhang ssDNA regions). An additional and surprising observation is the substantial stimulation of helicase activity by one of the *T.acidophilum* Cdc6 homologues. Cdc6 also stimulates duplex translocation by the helicase. These observations are in a sharp contrast to those made with MCM homologues from other archaea, where the helicase activity is inhibited when bound to Cdc6.

## MATERIALS AND METHODS

### Cloning and purification of the MCM, Cdc6-1 and -2 proteins

The gene encoding *T.acidophilum* MCM (Ta0799), Cdc6-1 (Ta0451m) and Cdc6-2 (Ta0636) were PCR amplified from genomic DNA using the primers shown in Supplementary Table 1 and cloned into the NdeI and BamHI (MCM), NheI and EcoRI (Cdc6-1) or NdeI and XhoI (Cdc6-2) sites of pET-21a (Novagene), with an in-frame six His-tag at the C-terminus. The proteins were overexpressed in *Escherichia coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene) and purified on a Ni-column as was previously described for the purification of the *M.thermautotrophicus* enzymes (6). The proteins were further purified to near homogeneity on a superose-6 gel-filtration column (HR10/30; GE Healthcare) equilibrated with 50 mM Tris–HCl (pH 8.0), 100 mM NaCl and 10% glycerol. These purified proteins (Supplementary Figure 1) were used for all the experiments described in this study. The proteins were aliquoted and frozen at −80°C.

A MCM protein containing a mutation at the nucleotide binding and hydrolysis site, substitution of Ala for Lys at residue 343 ($K_{343}A$), was generated using a PCR-based approach and the vector containing the wild-type gene as a template. The mutant protein was expressed and purified as described above for the wild-type enzyme.

### Gel-filtration analysis

One hundred micrograms of purified *T.acidophilum* or *M.thermautotrophicus* MCM were applied to a superose-6 gel-filtration column (HR10/30; GE Healthcare) pre-equilibrated with buffer containing 20 mM Tris–HCl (pH 7.5), 100 mM NaCl and 10% glycerol. Columns were run at 22°C.

### MCM helicase assay

Helicase substrates, with the sequences shown in Supplementary Table 1, were generated by complementary oligonucleotide hybridization and purified as described previously (7). The short DNA strand in each substrate was 5′-end-labeled using [γ-$^{32}$P]ATP and T4 polynucleotide kinase.

DNA helicase activity was measured in reaction mixtures (15 µl) containing 20 mM HEPES–NaOH (pH 7.5), 10 mM magnesium acetate, 3.3 mM ATP, 2 mM DTT, 0.1 mg/ml BSA, 10 fmol of $^{32}$P-labeled DNA substrate (3000 c.p.m./fmol) and proteins as indicated in the legend to Figure 2. After incubation at 60°C for 1 h, 5 µl 5× loading buffer (100 mM EDTA, 1% SDS, 0.1% xylene cyanol, 0.1% bromophenol blue and 50% glycerol) was added and aliquots were loaded onto a 10% polyacrylamide gel in 0.5× TBE (45 mM Tris, 45 mM boric acid and 1 mM EDTA) and electrophoresed for 1 h at 150 V. The helicase activity was visualized and quantified by phosphorimaging. All helicase experiments were repeated three to five times and their averages with standard deviations are shown in Figure 2 together with representative gels.

### Effect of Cdc6 on MCM helicase activity

The substrates to determine the effect of Cdc6 on MCM helicase activity and dsDNA translocation were made as described previously (7), using the oligonucleotides shown in Supplementary Table 1. The helicase substrates were made by annealing a 25mer (DF25F) or 61mer (DF61) oligonucleotide, which was pre-labeled with [γ-$^{32}$P]ATP and T4 polynucleotide kinase, to a 74mer oligonucleotide (DF74). The substrate for duplex DNA translocation assays was made by annealing a 61mer oligonucleotide (DF61), which was pre-labeled with [γ-$^{32}$P]ATP, to two other oligonucleotides: a 25mer (DF25) and a 50mer (DF50). DNA helicase activity was measured as described above using protein as indicated in the legends to Figures 3, 5 and 6. After incubation at 60°C for 1 h, reactions were stopped and analyzed as described above.

### Streptavidin displacement assay

Biotinylated oligonucleotides were labeled using [γ-$^{32}$P]ATP and T4 polynucleotide kinase at their 5′ end purified as previously described (7). Ten fmol of ss or ds oligonucleotides were incubated with 50 nM streptavidin (Rockland Immunochemicals) in a 15 µl helicase reaction mixture at 37°C for 5 min. The MCM helicase (2.7 pmol) was added together with 500 nM free biotin (Sigma) (to trap and sequester streptavidin if released by the helicase) in the presence or absence of Cdc6-2 protein (as indicated in the legend to Figure 6). After incubation for 1 h at 55°C, 5 µl of 5× loading buffer (0.1% xylene cyanol, 0.1% bromophenol blue and 50% glycerol) was added followed by electrophoretic analysis on a native 8% polyacrylamide gel in 0.5× TBE. The gels were analyzed using phosphorimaging.

### Two-hybrid analysis

For the two-hybrid analysis the genes encoding the *T.acidophilum* MCM (Ta0799), Cdc6-1 (Ta0451m) and Cdc6-2 (Ta0636) were PCR amplified from the pET-21a vectors containing the genes (described above) using the primers shown in Supplementary Table 1. The genes were cloned into the SalI and NotI sites of the pDBLeu vector (Invitrogen), resulting in a fusion protein with the GAL4 DNA binding domain (DB). MCM was also cloned into pPC86 (Invitrogen) using the same restriction sites, resulting in a fusion protein with the GAL4 activation domain (AD).

Plasmids encoding the AD and DB fusion proteins were co-transformed into yeast MaV203 cells (Invitrogen) according to the manufacturer's protocol. Cells were plated on complete supplement mixture (CSM) plates without Leu and Trp and grown for 3 days at 30°C. Colonies were streaked on CSM plates without Leu, Trp and His and containing 10 mM 3-amino-1,2,4-triazole to suppress glycerol phosphate dehydratase, an enzyme involved in histidine biosynthesis. Plates were immediately replica cleaned,

incubated at 30°C, and replica cleaned again after 24 h. Plates were incubated 2–3 days further before scoring.

### Protein pull-down assay

MCM proteins labeled with $^{35}$S were generated by *in vitro* transcription-translation using a wheat germ extract system (Promega) according to the manufacturer's protocol using $^{35}$S-Met. Following protein expression the proteins were purified on superose-6 gel-filtration columns (HR10/30; GE Healthcare) pre-equilibrated with 20 mM Tris–HCl (pH 7.5) and 100 mM NaCl. The fractions in which the $^{35}$S-MCM eluted were determined using SDS–PAGE analysis and autoradiography. As the amount of Met in the wheat extract is unknown, no specific activity and protein amount could be calculated.

Pull-down assays were carried out by incubating 1 µg of His-tagged Cdc6-1 or -2 protein with purified $^{35}$S-MCM protein in 500 µl buffer containing 20 mM Tris–HCl (pH 7.5) and 100 mM NaCl for 10 min at 25°C. The mixture was passed through a Ni-NTA spin column (Qiagen). Following binding the column was washed with reaction buffer. Bound protein was eluted with 200 µl elution buffer containing 20 mM Tris–HCl (pH 7.5), 100 mM NaCl and 500 mM imidazole. The presence of $^{35}$S-MCM was detected by 10% SDS–PAGE followed by phosphorimaging visualization. The eluted fractions were also quantitated using scintillation counting and compared to the amount loaded on the column.

## RESULTS

### The *T.acidophilum* MCM protein forms hexamers in solution

Studies on the MCM proteins from several archaea suggest that these proteins have different structures in different organisms. The MCM homologs of *S.solfataricus* (8,9), *A.fulgidus* (10) and *Methanococcoides burtonii* (11) form hexamers in solution while the *M.thermautotrophicus* enzyme appears to form dodecamers (12–14).

As a first step in characterizing the *T.acidophilum* MCM helicase, the aggregation form was determined. As shown in Figure 1 the protein forms hexamers in solution. In this regard, therefore, the protein is similar to the majority of the archaeal MCM proteins studied to date.

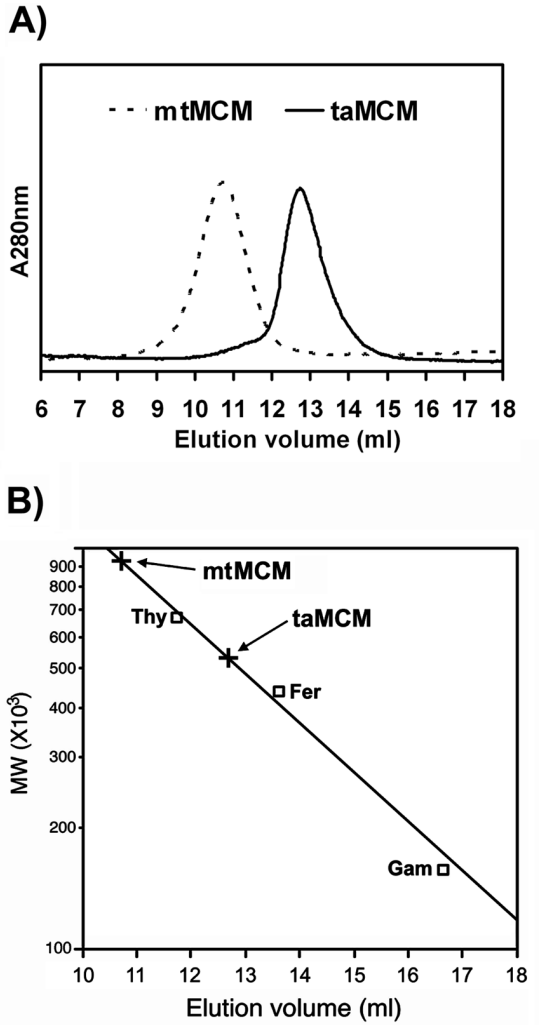


**Figure 1.** *T.acidophilum* MCM protein forms hexamers in solution. (**A**) An aliquot of 100 µg of purified *T.acidophilum* (taMCM) or *M.thermautotrophicus* (mtMCM) MCM protein was applied to a superose-6 gel-filtration column and analyzed as described in Materials and Methods. (**B**) The peak elution of the proteins in relation to the peak positions of thyroglobulin (Thy, 669 kDa), ferritin (Fer, 440 kDa), and gamma globulin (Gam, 158 kDa) is shown.

### MCM requires a forked DNA substrate for efficient DNA helicase activity

All archaeal MCM helicases studied to date were shown to be active on a flat substrate with a 3′-ssDNA overhang region [(1,2) and references therein]. Interestingly, the *T.acidophilum* MCM shows very limited helicase activity on a flat substrate containing a short duplex DNA region and only 3′-overhang ssDNA (Figure 2B and D). No helicase activity could be observed when the duplex region was >25 bp (data not shown). In addition, the sequence of the duplex has an influence on helicase activity as no activity could be detected when a G/C-rich 20 bp duplex with only a 3′-overhang region was used (data not shown), suggesting that the *T.acidophilum* MCM is a poor helicase on a substrate with only a 3′-overhanging region. Similar to all other archaeal MCM helicases the enzyme has a 3′–5′ directionality, as no activity was observed with substrate containing only a 5′-overhang region (Figure 2A and B) and only ATP and dATP could support helicase activity (data not shown).

In eukarya, it was shown that the Mcm4,6,7 complex has very poor helicase activity on substrates with only a 3′-overhang region, as the enzyme cannot unwind a duplex region >18 bp (15,16). The helicase activity of the Mcm4,6,7 complex is stimulated in the presence of forked DNA substrates (containing both 3′- and 5′-overhanging ssDNA) (15,16). Therefore, the activity of *T.acidophilum* MCM helicase on substrates containing a 25 bp duplex region and forked DNA structures of various lengths was determined (Figure 2). It was found that a fork structure stimulates the unwinding activity of the helicase with the major contribution by the extension of the 3′-overhang region (Figure 2A and C). Although short 5′-overhang ssDNA (up to 8 nt) stimulates

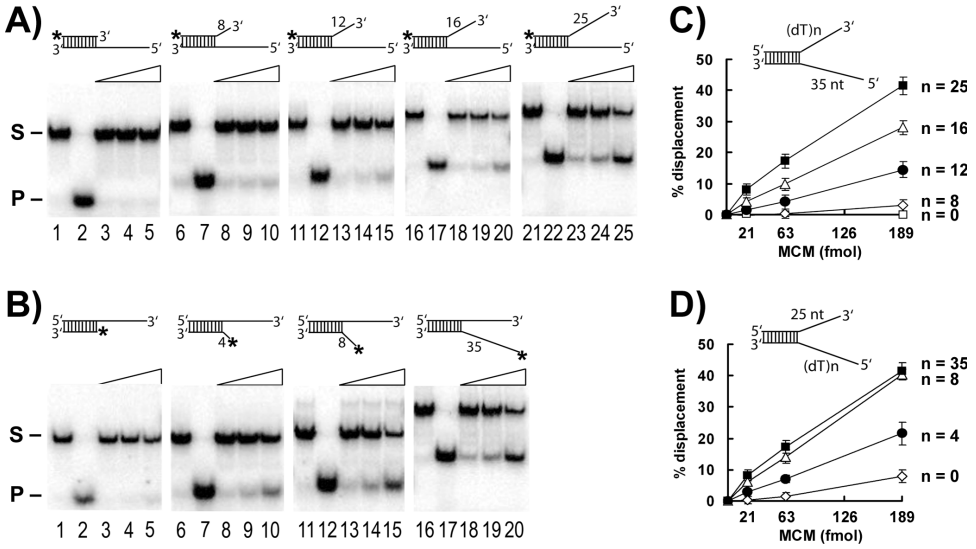


**Figure 2.** *T.acidophilum* MCM protein requires a forked DNA structure for efficient helicase activity. DNA helicase assays were performed as described in Materials and Methods using substrates with various 3′ (**A** and **C**) or 5′ (**B** and **D**) ssDNA overhang regions as indicated at the top of each panel. (A and B) Representative gels. Lanes 1, 6, 11, 16 and 21: substrate only; lanes 2, 7, 12, 17 and 22: boiled substrate; lanes 3, 8, 13, 18 and 23: 21 fmol; lanes 4, 9, 14, 19 and 24: 63 fmol; lanes 5, 10, 15, 20 and 25: 189 fmol of MCM (as hexamer). S, substrate; P, product. In (C and D), the average of three independent experiments with standard deviation is shown.

the helicase activity, longer overhang regions do not have an additional stimulatory effect (Figure 2B and D). These results suggest that the *T.acidophilum* MCM is similar to the eukaryotic Mcm4,6,7 complex by requiring a forked DNA structure for efficient helicase activity.

### Cdc6-2 protein stimulates MCM helicase activity

Studies with the Cdc6 proteins from a number of archaea have shown that the proteins inhibit the helicase activity of their respective MCM proteins (17–19). It was also found that the inhibition is species specific (17) and requires direct protein-protein interaction between Cdc6 and MCM (19). Thus the effect of the two *T.acidophilum* Cdc6 proteins on MCM helicase activity was determined.

Surprisingly, Cdc6-2 dramatically stimulated the helicase activity of MCM (Figure 3A and B lanes 8–10) with substrate containing only a 3′-ssDNA overhang region (Figure 3A) or a fork-like structure (Figure 3B). No helicase activity could be detected when a mutant MCM protein ($K_{343}A$) lacking helicase activity was used (Figure 3A and B lane 11), demonstrating that the results observed are due to the direct effect of Cdc6-2 protein on MCM and not due to a helicase-like activity by Cdc6-2. Cdc6-1, on the other hand, has no effect on MCM helicase activity, either stimulatory or inhibitory (Figure 3A and B lanes 4–6).

### Cdc6-2 interacts with MCM protein

The stimulatory effect of Cdc6-2 on MCM helicase activity suggests a direct interaction between the proteins. Therefore two-hybrid and pull-down analyses were performed to study the interactions between *T.acidophilum* MCM and the two Cdc6 proteins (Figure 4). For the two-hybrid analysis the MCM self-interaction was used as a control, as the molecule forms a hexamer (Figure 1). The two-hybrid analysis (Figure 4A) and the pull-down assay (Figure 4B) revealed that MCM interacts only with Cdc6-2 protein and no interaction could be detected with Cdc6-1. Some non-specific binding by MCM to the nickel resin is noted (Figure 4B lane 2) and expected, as the column was washed with only 100 mM NaCl. It is known that closer to 500 mM NaCl is needed to eliminate non-specific binding, but this high salt concentration would effect MCM interaction with Cdc6. The results suggest that the stimulation of MCM helicase activity by Cdc6-2 is due to the interactions between them. The inability of Cdc6-1 to affect the helicase activity (by either stimulation or inhibition) can be explained by the inability of the protein to interact with MCM. It was previously shown that direct Cdc6-MCM interactions are needed for the inhibitory effect of the *M.thermautotrophicus* Cdc6 on MCM helicase activity (19).

### Cdc6-2 protein stimulates dsDNA translocation by MCM

In addition to ssDNA translocation, the *M.thermautotrophicus* MCM and the eukaryotic Mcm4,6,7 complex were also shown to move along duplex DNA (7,20). dsDNA translocation by the archaeal and eukaryal enzymes, however, required different DNA substrates. While the archaeal MCM can initiate dsDNA translocation from a flat duplex without a 3′-overhang, the eukaryotic enzyme required a 3′-overhanging region (7).

Thus, the ability of the *T.acidophilum* MCM to translocate along dsDNA, and the effect of Cdc6-2 on this activity, was determined using a similar approach to that previously used to study the *M.thermautotrophicus* and *Schizosaccharomyces pombe* MCMs (7). As shown in Figure 5A and C the enzyme is capable of moving along the duplex because it unwinds the duplex on the right side of the substrate. No unwinding

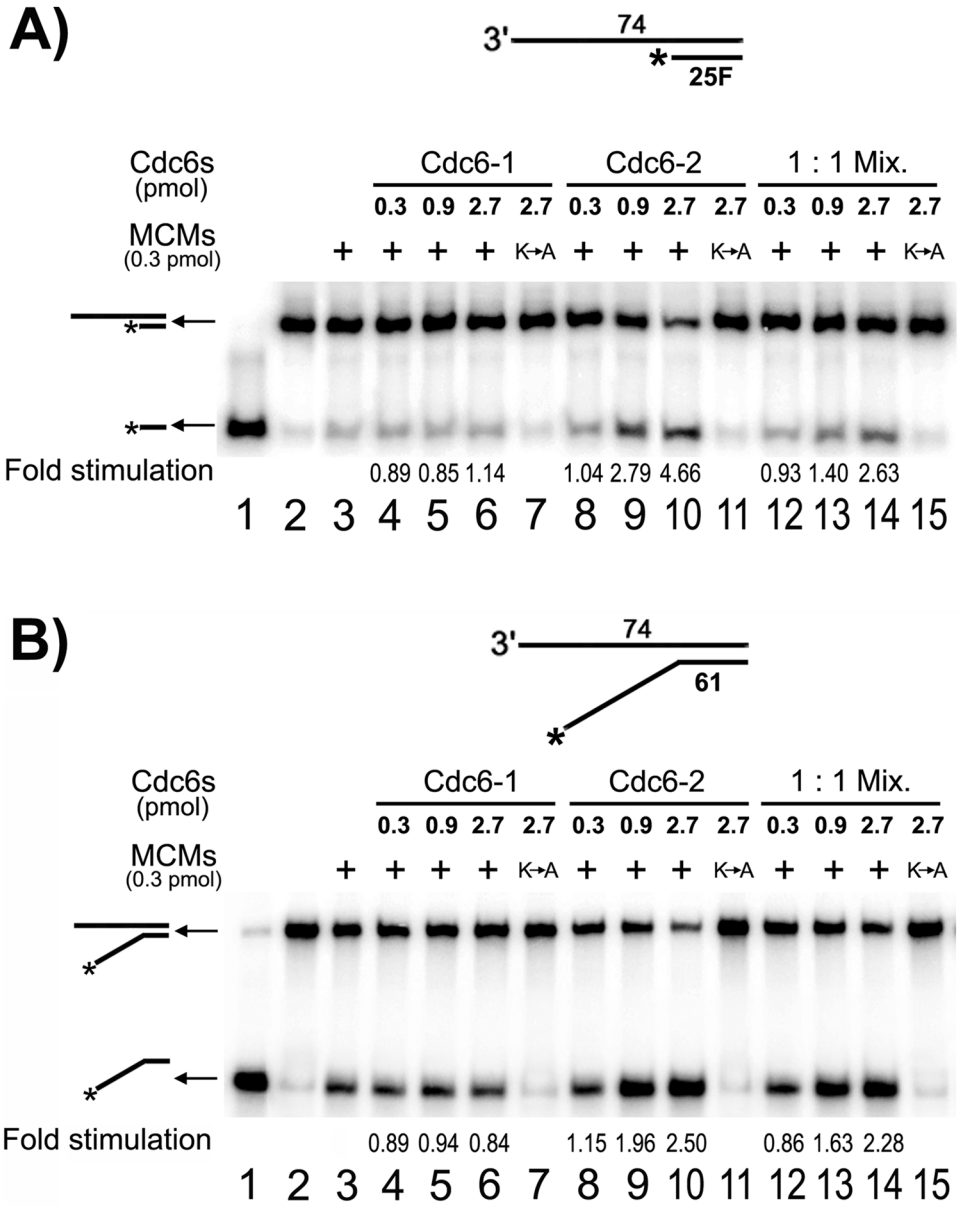


**Figure 3.** *T.acidophilum* Cdc6-2 protein stimulates MCM helicase activity. DNA helicase assays were performed as described in Materials and Methods using 0.3 pmol of MCM as hexamer (lanes 3–6, 8–10 and 12–14) or MCM $K_{343}A$ mutant (lanes 7, 11 and 15) with increasing amounts of Cdc6-1 (lanes 4–7), Cdc6-2 (lanes 8–11) or a 1:1 mixture of both proteins (lanes 12–15). The fold stimulation of helicase activity in comparison to reactions without Cdc6 proteins (lane 3) is shown. Lane 1, boiled substrate; lane 2, substrate only. A, flat substrate; B, forked substrate.

could be observed when a mutant protein ($K_{343}A$) lacking helicase activity was used (Figure 5A, lane 6).

To date, although dsDNA translocation was reported with the replicative helicases of all three domains of life (7,20), *M.thermautotrophicus* is the only archaeal MCM for which duplex DNA translocation has been shown. In light of the data presented here, one may expect it to be a general phenomenon for other archaeal MCM enzymes.

As Cdc6-2 was shown to substantially stimulate the *T.acidophilum* MCM helicase activity (Figure 3), the effect of the protein on dsDNA translocation was also determined. As shown in Figure 5B and C, the Cdc6-2 protein also substantially stimulates the duplex translocation by MCM. The results observed are due to helicase activity, and not indirectly by the presence of Cdc6 as no activity could be detected when a mutant MCM protein ($K_{343}A$) without helicase activity was used (Figure 5B, lane 6).

However, the assay described above used indirect evidence to demonstrate duplex translocation by MCM. Thus, the Cdc6-2 stimulatory effect may not be on duplex movement but rather on the unwinding of the right-side duplex. Therefore, a different approach was used to directly demonstrate the effect of Cdc6-2 on dsDNA translocation by MCM. It was shown that the *M.thermautotrophicus* MCM is capable of displacing streptavidin from biotinylated oligonucleotides while translocating along ss or dsDNA (7). The experiments were performed using biotinylated oligonucleotides that were pre-bound by streptavidin. The helicase was incubated with

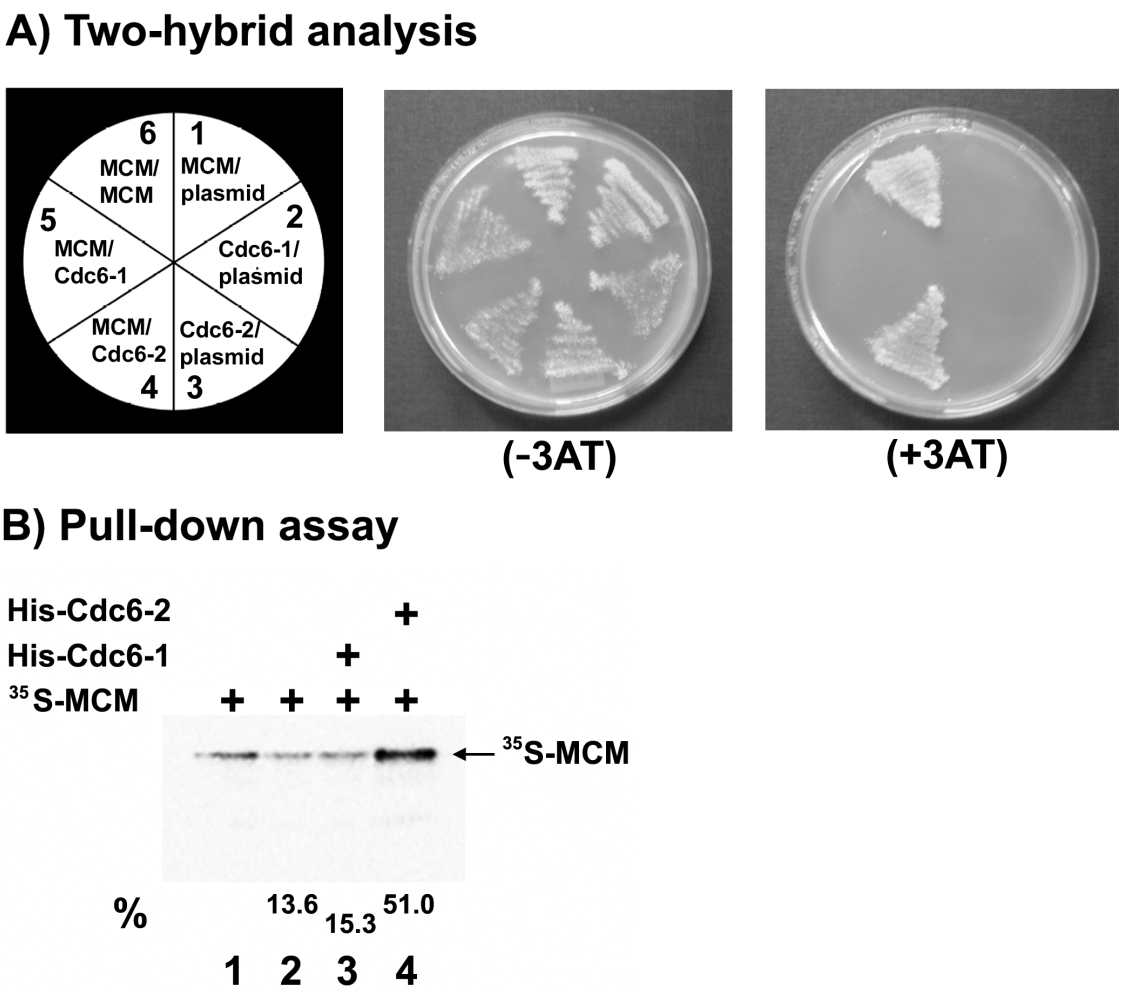


**Figure 4.** *T.acidophilum* Cdc6-2 protein interacts with MCM. (**A**) Two-hybrid analysis of the interactions between MCM and the Cdc6 proteins were performed as described in Materials and Methods. The left panel shows an outline of the plate. 1, pDBLeu-TaMCM and pPC86; 2, pDBLeu-TaCdc6-1 and pPC86, 3, pDBLeu-TaCdc6-2 and pPC86; 4, pDBLeu-TaCdc6-2 and pPC86-TaMCM; 5, pDBLeu-TaCdc6-1 and pPC86-TaMCM; 6, pDBLeu-TaMCM and pDBLeu-TaMCM. Center panel, CSM plate minus Leu, Trp and His; Right panel, CSM plate minus Leu, Trp and His containing 10 mM 3-amino-1,2,4-triazole (3AT). (**B**) Protein pull-down analysis of the interactions between MCM and the Cdc6 proteins were performed as described in Material and Methods by binding $^{35}$S-MCM and His-tagged Cdc6-1 or -2 protein followed by absorption to Ni-column. Lane 1, 20% of the $^{35}$S-MCM used for each binding reaction; lane 2, $^{35}$S-MCM alone; lane 3, $^{35}$S-MCM and His-Cdc6-1; lane 4, $^{35}$S-MCM and His-Cdc6-2. The percent of the input $^{35}$S-MCM retained on the column is noted below each lane.

the substrate in the presence of a large excess of biotin to serve as a trap to bind streptavidin upon its displacement from the DNA by the helicase. A similar approach was used to determine whether the *T.acidophilum* Cdc6-2 could stimulate MCM translocation along ss and dsDNA. As shown in Figure 6, there is little displacement of streptavidin from ss or dsDNA by the *T.acidophilum* MCM (Figure 6A and B, lane 2). In the presence of Cdc6-2, however, streptavidin displacement was stimulated (Figure 6A and B, compare lanes 3–5 to lane 2). To determine whether the activity observed is due to the effect on MCM and not indirectly by the presence of Cdc6-2, a mutant MCM ($K_{343}A$) devoid of helicase activity was used in the presence of high concentrations of Cdc6-2. No streptavidin displacement could be observed under these conditions (Figure 6A and B, lane 6).

Taken together, these studies suggest that the Cdc6-2 protein directly stimulates MCM movement along ss and dsDNA.

## DISCUSSION

Archaea are adapted to a variety of different environments, and they often thrive in habitats with extreme physical or chemical characteristics, e.g. extreme temperatures, pH or osmotic pressure. Genome analysis of a large number of archaea shows that different species contain diverse sets of enzymes. Thus, in order to understand the diversity and properties of the replication machinery in this domain one has to study a large number of organisms with diverse phylogenetic affiliations and/or environmental growth conditions. To date, the replication machinery of *M.thermautotrophicus*, *S.solfataricus* and *A.fulgidus* are the most extensively studied. Although the archaeal MCM proteins are homologous to each other each exhibits some unique biochemical properties. The study described here reveals several features of the *T.acidophilum* MCM that have not yet been reported for an archaeal enzyme.

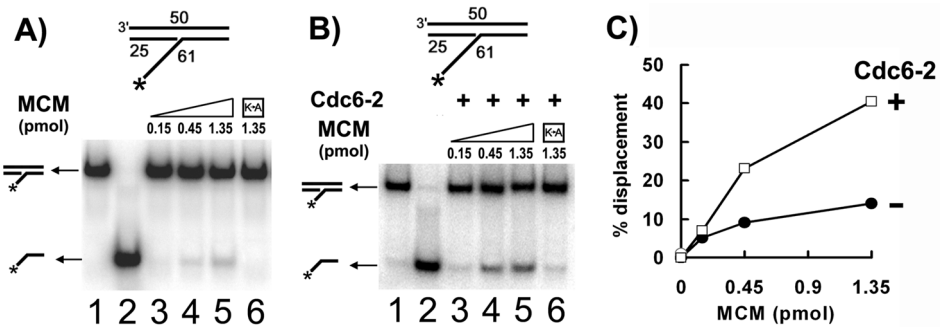


**Figure 5.** *T.acidophilum* Cdc6-2 protein stimulates MCM dsDNA translocation. Shown is a MCM helicase assay performed as described in Materials and Methods in the absence (**A**) or presence (**B**) of Cdc6-2 protein (2.7 pmol) and increasing amounts of MCM protein. Panels A and B, lane 1, boiled substrate; lane 2, substrate only; lane 3, 0.15 pmol of MCM; lane 4, 0.45 pmol of MCM; lane 5, 1.35 pmol of MCM; lane 6, 1.35 pmol of MCM $K_{343}A$ mutant as hexamer. Panel C, a summary of the data.

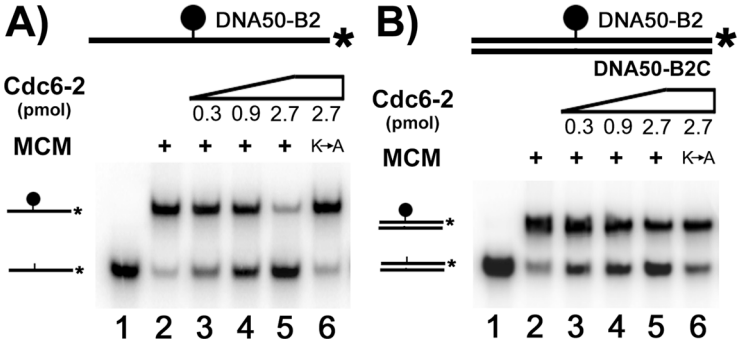


**Figure 6.** *T.acidophilum* Cdc6-2 protein stimulates streptavidin displacement from biotinylated oligonucleotide by the MCM helicase. Helicase-mediated displacement of streptavidin from streptavidin-biotinylated ssDNA (**A**) and dsDNA substrates (**B**) was tested as described in Materials and Methods with 2.7 pmol of MCM (lanes 2–5) or MCM $K_{343}A$ mutant as monomer in the presence of increasing amounts of Cdc6-2. Lane 1, substrate without streptavidin; lane 2, no Cdc6-2; lane 3, 0.3 pmol; lane 4, 0.9 pmol; lanes 5 and 6, 2.7 pmol of Cdc6-2 protein.

One of the differences between the *T.acidophilum* MCM helicase and other archaeal MCMs studied to date is its poor activity on a substrate containing only a 3′-ssDNA overhang. The other archaeal enzymes possess a robust helicase activity on such a substrate. The *T.acidophilum* enzyme requires a forked DNA structure for efficient helicase activity. This observation is reminiscent of the situation with the eukaryotic MCM. It was shown that the eukaryotic Mcm4,6,7 complex cannot displace a duplex region >18 nt if provided only with a 3′-ssDNA overhang region (15,16). The activity is dramatically stimulated in the presence of a forked DNA structure containing both 3′- and 5′-overhang ssDNA (15,16). Thus, although all archaeal MCM proteins contain biochemical properties similar to the eukaryotic Mcm4,6,7 complex, including 3′–5′ helicase activity and DNA-dependent ATPase activity, the *T.acidophilum* enzyme behaves more like the eukaryotic enzymes *in vitro* due to its requirement for a fork-like structure for efficient activity. It is important to note that the Mcm4,6,7 complex may not be the active eukaryotic helicase (it is possible it is the Mcm2–7 complex), as all six MCM subunits are essential to viability. In addition, it is likely that *in vivo* the helicase is aided by other proteins that enable it to efficiently unwind different substrates. It was shown that the eukaryotic helicase is a part of a large complex which includes the GINS complex and Cdc45.

However, even in the presence of forked substrate the *T.acidophilum* MCM helicase activity is relatively poor in comparison to that reported for other archaeal MCM proteins. The investigation of this poor activity led to the discovery of another feature of the *T.acidophilum* initiation proteins that has not yet been reported for any other archaeal system. It was found that one of the Cdc6 homologues dramatically stimulates the MCM helicase activity.

The archaeal Cdc6 protein has been suggested to function as the helicase loader and thus to be the functional homologue of the bacterial helicase loader, DnaC [(1) and references therein]. In bacteria, when DnaC binds to the helicase, DnaB, it inhibits helicase activity. Only after proper assembly of the helicase at the origin does DnaC dissociate from DnaB, allowing it to function as a helicase. In support of the hypothesis that the archaeal Cdc6 may have a similar function, it was shown that the two Cdc6 proteins from *M.thermautotrophicus* (17) and the three *S.solfataricus* homologues (18,21) inhibit their respective MCM helicase activities. Thus, it was unexpected to find that the *T.acidophilum* Cdc6-2 protein stimulates, rather than inhibits, the MCM helicase activity. The mechanism by which the Cdc6 protein stimulates the activity is currently unknown. It is possible that the presence of Cdc6 stabilizes the interactions between MCM and DNA. Alternatively, Cdc6 may alter the structure of MCM. In eukarya, the Mcm4,6,7 complex

forms double hexamers on forked structures (15). In archaea, both the *M.thermautotrophicus* and *S.solfataricus* proteins form dodecamers in solution and have been proposed to function as double hexamers (12,13,22). Thus, it may be that in *T.acidophilum* the Cdc6-2 protein is required to form and/or stabilize MCM dodecamers from the hexameric structures. Regardless of the mechanism of stimulation, it is not yet known why the Cdc6-2 protein from *T.acidophilum* exhibits a property that is different from that of other archaeal Cdc6 proteins and future studies are needed to address this question.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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