Biochemical and Mutational Analyses of a Unique Clamp Loader Complex in the Archaeon *Methanosarcina acetivorans*

Yi-Hsing Chen, Svetlana A. Kocherginskaya, Yuyen Lin, Binjon Sriratana, Angelica M. Lagunas, Justin B. Robbins, Roderick I. Mackie, and Isaac K. O. Cann

From the Department of Animal Sciences and the Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Clamp loaders orchestrate the switch from distributive to processive DNA synthesis. Their importance in cellular processes is underscored by their conservation across all forms of life. Here, we describe a new form of clamp loader from the archaeon *Methanosarcina acetivorans*. Unlike previously described archaeal clamp loaders, which are composed of one small subunit and one large subunit, the *M. acetivorans* clamp loader comprises two similar small subunits (*M. acetivorans* replication factor C small subunit (MacRFCS)) and one large subunit (MacRFCL). The relatedness of the archaeal and eukaryotic clamp loaders (which are made up of four similar small subunits and one large subunit) suggests that the *M. acetivorans* clamp loader may be an intermediate form in the archaeal/eukaryotic sister lineages. The clamp loader complex reconstituted from the three subunits MacRFCS1, MacRFCS2, and MacRFCL stimulated DNA synthesis by a cognate DNA polymerase in the presence of its sliding clamp. We used site-directed mutagenesis in the Walker A and SRC motifs to examine the contribution of each subunit to the function of the *M. acetivorans* clamp loader. Although mutations in MacRFCL and MacRFCS2 did not impair clamp loading activity, any mutant clamp loader harboring a mutation in MacRFCS1 was devoid of the clamp loading property. MacRFCS1 is therefore critical to the clamp loading activity of the *M. acetivorans* clamp loader. It is our anticipation that the discovery of this unique replication factor C homolog will lead to critical insights into the evolution of more complex clamp loaders from simpler ones as more complex organisms evolved in the archaeal/eukaryotic sister lineages.

As the molecular complexes that switch DNA polymerases from distributive to processive DNA synthesis, clamp loaders are critical to the evolution of large genomes. Replicative DNA polymerases are incapable of de novo DNA synthesis. Therefore, they require initial synthesis of primers by a specialized protein called DNA primase. After playing its role, the primase is displaced by a clamp loader, which couples ATP hydrolysis to loading of a ring-shaped sliding clamp onto the DNA template (1). Through physical interaction with its cognate DNA polymerase, the sliding clamp enables the polymerase to achieve the high speed DNA synthesis required for replication by tethering the DNA polymerase onto the template (2). The critical functions of sliding clamps and clamp loaders are underscored by their conservation across the three domains of life and also in bacteriophage such as T4 phage (3). In eukaryotes, the clamp loader, also known as replication factor C (RFC), is made up of four small (RFCs) subunits and one large subunit (RFCL). The four RFCs proteins are very similar in amino acid sequence, and although they seem redundant, each subunit is essential for cell proliferation (5). Previous analyses have shown that archaeal clamp loaders are related to their eukaryotic functional homologs. However, all hitherto reported archaeal clamp loaders comprise one RFC subunit and one RFCL subunit (6–9). Interestingly, in the archaeal clamp loaders, the single RFC subunit oligomerizes to mimic the four different RFCs subunits in eukaryotes (8–10). Thus, eukaryotes have a pentameric clamp loader made up of five different proteins (11), whereas the archaeal functional homolog is a pentameric complex of two different proteins (9, 12).

The striking similarities among the eukaryotic RFCs proteins suggest that they evolved from a single ancestral gene. However, evidence for how this might have occurred has been elusive. Our effort to systematically assemble the proteins required for replicating the genome of *Methanosarcina acetivorans*, a mesophilic species of Archaea, has led to several findings of interest to evolution of DNA replication proteins in the archaeal/eukaryotic sister lineages (13–15). Here, we present the results from biochemical and mutational analyses of an unusual clamp loader found in Methanosarciinales. This clamp loader type (composed of two RFCs subunits and one RFCL subunit) may represent a critical link in the evolution of complex clamp loaders from simple forms in the archaeal/eukaryotic sister lineages. In addition to the RFC complex, each subunit of the methanosarcinal RFC homolog was expressed as an individual protein and biochemically characterized. Furthermore, we used mutational analysis of residues involved in ATP binding and hydrolysis to study the contribution of each subunit to the function of the *M. acetivorans* clamp loader. Nucleotide binding and hydrolysis are critical to loading of the sliding clamp by the clamp loader. Clamp loaders are members of the AAA+ (ATPases associated with a variety of cellular activities) ATPase family, and their ATP-binding sites are located at subunit interfaces (10, 11, 16, 17). Critical to nucleotide binding and hydrolysis in these proteins are the P-loop (Walker A motif) and an SRC motif from an adjacent subunit (11, 16). Thus, we targeted critical residues in the P-loop and SRC motif for site-directed mutagenesis.

The organisms in which the clamp loader type described in this report is found are quite interesting among the species of Archaea.
Halobacterium sp. NRC-1 harbors a genome of ~2.0 Mb in size, and in addition, it has two large extrachromosomal replicons of 350 and 200 kb (18). Members of the order Methanosarcinales have the largest known archaenal genomes (4.1–5.7 Mb) (19, 20). Furthermore, they are unique among the species of Archaea in forming complex multicellular structures (21). The discovery of this new form of RFC in these organisms is of major importance, as further biochemical and genetic analyses may provide us with critical insights into cellular developments that might have led to a requirement for more complex clamp loaders from the equally competent but simple ones found in the archaeal/eukaryotic sister lineages.

**MATERIALS AND METHODS**

**Construction of Plasmids for Overproduction of RFC Proteins—** During annotation of the genome sequence of the mesophilic archaen M. acetivorans (Mac), we discovered two genes coding for two different RFC subunits designated MacRFC1 and MacRFC2 and another gene coding for one RFC1 subunit (MacRFC1). To express the individual genes for biochemical analysis, they were placed in-frame with the His6-encoding sequence of a modified PET28a plasmid (Novagen). The modification in the PET28a plasmid was a replacement of the kanamycin resistance gene with the ampicillin resistance gene (14). The individual genes were also placed in a PET21a plasmid (Novagen) for expression as proteins without a His6 tag. The plasmid constructs were thus designated PET28/rfcs1, PET28/rfcs2, and PET28/rfcl for the PET28a derivatives and PET21/rfcs1, PET21/rfcs2, and PET21/rfcl for the PET21a derivatives. In the experiments in which genes were coexpressed, the PET28a construct and a second plasmid, pACYCDuet (Novagen), were used except for the coexpression of rfcs1/rfcs2, which was carried out in pACYCDuet alone. Table 1 shows the pACYCDuet plasmid allows insertion of one or two genes for expression. The plasmids for coexpression of the three genes in different combinations were therefore PET28/rfcl-pACYCDuet/rfcs1, PET28/rfcl-pACYCDuet/rfcs2, pACYCDuet/rfcs1/rfcs2, and PET28/rfcl-pACYCDuet/rfcs1/rfcs2. The PET28a constructs contained an ampicillin resistance gene, and the pACYCDuet construct contained a chloramphenicol resistance gene; therefore, the medium for coexpression contained the two antibiotics for selection. All DNA inserts were sequenced (W. M. Keck Center for Comparative and Functional Genomics, University of Illinois at Urbana-Champaign) to ensure the integrity of the gene. The oligonucleotides used for amplification of the genes expressed in this study are shown in TABLE ONE.

**Site-directed Mutagenesis—** The conserved lysines and arginines located in the Walker A (RFC box III) and SRC (RFC box VII) motifs, respectively (see Fig. 1), of the RFC subunits were mutated to alanine. The RFC1 subunit lacks an SRC motif. Therefore, only the lysine in the Walker A motif was mutated to alanine. The mutations were carried out with the QuikChange® multisite-directed mutagenesis kit (Stratagen). The primers for the mutagenesis are shown in TABLE ONE. To allow cloning of both RFC1 subunits into pACYCDuet, an Ncol site in MacRFC1 was abolished through site-directed mutagenesis with primer RFCS1Ncol (TABLE ONE), which introduced only a silent mutation into the gene. The gene for MacRFC1 was cloned with Ndel and Xhol, and the gene for MacRFC2 was digested with Ncol and Xhol and cloned into the Ncol/Sall site in pACYCDuet. The genes for the mutant subunits were used in different combinations to create different mutants of the MacRFC complex.

**Production of Recombinant Proteins—** Plasmids harboring each individual RFC gene were transformed into Escherichia coli BL21-Codon-Plus(DE3)-RII cells (Stratagen) and spread on LB plates containing 100 μg/ml ampicillin and 50 μg/ml chloramphenicol. After an overnight incubation, a single colony was picked from each plate, inoculated into LB broth containing the two antibiotics at the concentrations mentioned above, and incubated with vigorous shaking at 37 °C until the absorbance at 600 nm reached 0.3. Gene expression was induced by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside; the incubation temperature was dropped to 16 °C; and cells were cultured for 12 h. One liter of culture from each cell line was centrifuged to pellet the cells. The cells were suspended in a lysis buffer or in buffer A (50 mM sodium phosphate (pH 7.0) and 300 mM NaCl) and lysed using a French pressure cell (American Instruments Co.) to release the cell contents. Because both MacRFC1 and MacRFC2 were produced with an N-terminal His6 tag, the supernatants for MacRFC1 and MacRFC2 were each applied to a cobalt affinity resin (TALON™, Clontech) to immobilize the His6-tagged proteins (His6-MacRFC1 and His6-MacRFC2). The resin was washed extensively with the lysis buffer, and the bound protein was eluted with buffer A containing 150 mM imidazole. Aliquots of the samples were resolved by SDS-PAGE, and the fractions containing the respective RFC subunit were pooled together, suspended in buffer B (50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 500 mM NaCl, 1 mM dithiothreitol (DTT), and 20% glycerol), and dialyzed against the same buffer. The samples were then loaded onto a Superose 12 HR 10/30 gel filtration column (Amersham Biosciences) equilibrated with buffer B. The chromatography was developed with buffer B at a flow rate of 0.35 ml/min, and 0.5-ml fractions were collected for analysis by SDS-PAGE.

In the case of MacRFC1, the plasmid construct was transformed into E. coli BL21(DE3) cells (Stratagen). The production of the MacRFC complex and all of its derivatives and also all other combinations of the MacRFC subunits tested was carried out in E. coli BL21(DE3) cells. The cells carrying MacRFC1 were selected on ampicillin-containing LB medium, whereas the cells carrying MacRFC1/MacRFC2 were selected on chloramphenicol-containing LB medium. The RFC complex and its derivatives were selected on ampicillin- and chloramphenicol-containing LB medium. Therefore, His6-MacRFC1 and the MacRFC complex (coexpressed His6-RFCL, RFC1, and His6-RFC2) were purified by affinity chromatography on cobalt affinity resin as described above, followed by passage through a heparin column and then gel filtration chromatography. For affinity chromatography on a HiTrap™ HP column (5 ml; Amersham Biosciences), the elution buffer contained 500 mM imidazole. In the heparin chromatography step, His6-RFCL or the MacRFC complex obtained from the affinity chromatography was dialyzed against buffer C (50 mM KH2PO4 (pH 6.8), 100 mM NaCl, 7 mM β-mercaptoethanol, and 10% glycerol) and then loaded onto HiTrap™ heparin HP column (5 ml; Amersham Biosciences) equilibrated with the same buffer. The column was washed with 5 column volumes of buffer C, followed by elution of bound proteins with buffer C containing 1 M NaCl. The fractions containing the proteins were dialyzed against buffer B and applied to a gel filtration column as described above for the RFC subunits. His6-RFCL and the MacRFC complex were each stored in buffer containing 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5 mM MgCl2, 10% glycerol, and 1 mM DTT until used. The gene for a homolog of the eukaryotic proliferating cell nuclear antigen (PCNA) in M. acetivorans was cloned into pET21a for expression. The expression and purification of MacPCNA were performed following a method described previously (22) for Pyrococcus furiosus (Pfu) PCNA, except for the omission of the heating step because MacPCNA is from a mesophilic organism. In brief, this method involved Polymin P treatment for 30 min at 4 °C, ammonium sulfate precipitation, and anion exchange chromatography (HiTrap Q, Amersham Biosciences). The cloning, expression, and purification of M. ace-
**Oligonucleotides used in this study**

The oligonucleotides for genes were used to clone *M. acetivorans* rfc1, rfc2, rfc1, and PCNA, respectively. The RFC31ΔNcol mutagenesis oligonucleotide was used to create a silent mutation to allow cloning of rfc1 in pACYCDuet. The other mutagenesis oligonucleotides were used to create site-directed mutations in the putative Walker A and SRC motifs in the MacRFC genes. The truncation oligonucleotides were used to delete the PIP boxes of MacPolBI. The ATPase assay oligonucleotides were used as effectors in the ATPase activity analysis. The electrophoretic mobility shift assay (EMSA) and primer extension oligonucleotides were used for ssDNA binding and DNA synthesis analyses, respectively. Restriction sites are underlined.

| Oligonucleotide | Nucleotide sequence |
|----------------|---------------------|
| **rfcs1 gene** |  |
| MacRFCFS1F | 5’-AAAAAACATATGCGACCATATATGAGGAGAGTCTTAAGG |
| MacRFCFS1R | 5’-TTTTTTCGAGTCTAGTTTCTCTGTTTATATG |
| **rfcs2 gene** |  |
| MacRFCFS2F | 5’-AAAAAACATATGGGATATCGAATGGGAGATTTGTTT |
| MacRFCFS2R | 5’-TTTTTTCGAGTCTAGTTTCTCTGTTTATATG |
| **rfcl gene** |  |
| MacRFCLF | 5’-AAAAAACATATGAGTTCGCGAATGGGAGATTTGTTT |
| MacRFCLR | 5’-TTTTTTCGAGTCTAGTTTCTCTGTTTATATG |
| **PCNA gene** |  |
| MacPCNAF | 5’-AAAAAACATATGTTCAAGCGACATTATATGCGAG |
| MacPCNAR | 5’-TTTTTTCGAGTCTAGTTTCTCTGTTTATATG |
| **Mutagenesis** |  |
| RFC31ΔNcol | 5’-CGCAGTCCGCGAGAATTTGGAAGGTTGCTAG |
| RFC31(K59A) | 5’-CTTCCGCGGTTGCAAGGACACAGGCTCGAGTT |
| RFC32(K52A) | 5’-CTGGAAAGCCTGGAGAAGAGGCTCGAGTT |
| RFC32/K56A | 5’-CTGCGGCGGATAGGAGCGACTTCAAGTGCTCAT |
| RFC31(R161A) | 5’-ATTGAGGCCATTTCAGTGCGGTGTTGTTACAGG |
| RFC32(R180A) | 5’-ATTTCCTCCCCCTCGTACGCGGGGAGCTAGTTT |
| **Truncation** |  |
| PolBIAC1 | 5’-GGCGGGCGGTCTTTACGCAACGCGCGTTTCTCGGAAGCTAGCAAG |
| PolBIAC2 | 5’-GGCGGGCGGTCTTTACGCAACGCGCGTTTCTCGGAAGCTAGCAAG |
| **ATPase assay** |  |
| ssDNA | 5’-GGAAAAAACAGGGAGAGGCAATCGGATTTACAGTAGTAT |
| dsDNA | A T. *polyaccharolyticum* carbohydrate-binding module gene (375 bp) |
| pDNA primer | 5’-CCGTCAGTTCGTGTTTTGAAACAGCCCGCAGTGAATT |
| pDNA template | 5’-GTACCGGAGCCGGTTTACTCAGGTTTCTCAGGTTT |
| **EMSA** |  |
| CDC6-1F | 5’-AAAACATATGAAATGGAAGCTCAGTACGTTGTTT |
| **Primer extension** |  |
| M13-(6205–6234) | 5’-ATTTCCCCCTCGTACGCGGGGAGCTAGTTT |

*tivorans* DNA polymerase BI (PolBI) and its truncated forms were as described in our previous reports (14, 15). The primers for the C-terminal truncation of MacPolBI are shown in TABLE ONE. All of the mutant MacRFC proteins were expressed and their products were purified as described for their wild-type counterparts.

**Western Blot Analysis**—Polyclonal antibodies were raised against purified recombinant MacRFC51 and MacRFC52 (Immunological Resource Center, University of Illinois at Urbana-Champaign). In the case of His<sub>6</sub>-MacRFC1 and His<sub>6</sub>-MacRFC2, an antibody commercially available for detection of the His<sub>6</sub> tag (Amersham Biosciences) was purchased and used according to the manufacturer’s instructions. All protein samples of interest were resolved by SDS-PAGE, followed by electroblotting onto polyvinylidene difluoride membrane (Hybond<sup>TM</sup>-P, Amersham Biosciences), and analyzed as described previously (13).

**Estimation of Subunit Organization by Gel Filtration**—To estimate the subunit organization of individual proteins and protein complexes in solution, the RFC proteins were subjected to gel filtration analysis. Purified proteins were dialyzed against buffer B. Samples were then loaded onto a Superose 12 HR 10/30 gel filtration column equilibrated with buffer B. The chromatography was developed with buffer B at a flow rate of 0.35 ml/min, and 0.5-ml fractions were collected and analyzed by SDS-PAGE. The column was calibrated by running a set of protein standards (thyroglobulin, 669 kDa; ferritin, 440 kDa; aldolase, 158 kDa; catalase, 58 kDa; ovalbumin, 43 kDa; and ribonuclease A, 13.7 kDa) under the same conditions.

**Electrophoretic Mobility Shift Assay**—The MacRFC complex and its individual subunits were tested for their ability to bind to single-stranded DNA (ssDNA) and singly primed ssDNA (pDNA). The pDNA was made by end labeling the pDNA primer (38-mer) and annealing it to the partially complementary pDNA template (76-mer). The nucleotide sequences of the primer and template are shown in TABLE ONE. Increasing amounts of proteins were incubated with 0.5 pmol of γ<sup>32</sup>P-end-labeled ssDNA (CDC6-1F (40-mer oligonucleotide) in TABLE ONE) or γ<sup>32</sup>P-labeled pDNA in 10 μl of binding buffer (20 mM Tris acetate (pH 8.0) and 0.5 mM magnesium acetate) at 37 °C for 30 min. Two microliters of loading dye (20 mM Tris acetate (pH 8.0) 10% glycerol, and 0.1% bromophenol blue) were added to the product of the reaction and resolved by 1% agarose gel electrophoresis in 0.1X Tris acetate/EDTA buffer (15). The gels were dried, and the products were visualized by autoradiography.

**ATPase Activity**—ATPase activity was measured in a reaction mixture (20 μl) containing 25 mM Tris-HCl (pH 7.5), 0.1 mM DTT, 6 mM...
MgCl₂, 0.5 μg/μl bovine serum albumin, 0.034 μM [γ-32P] ATP (6000 Ci/mmol), 10 μM unlabeled ATP, and one of the following: 2.4 μg of MacRFCS1, 1.2 μg of MacRFCS2, 2.1 μg of MacRFCL, or 0.45 μg of MacRFC complex. The reaction mixture was incubated at 37 °C for 40 min and then terminated by addition of 2 μl of a 400 mM EDTA solution. When dATPase activity was investigated, the radioactive and nonradioactive ATPs were substituted with radioactive and nonradioactive dATPs, respectively. One microliter of products from each reaction mixture was spotted on polyethyleneimine-cellulose thin layer plates (Merck, Darmstadt, Germany) and then developed in a solution containing 1.0 M formic acid and 0.5 M LiCl for 10 min at room temperature. When their effects were assessed, cofactors or effectors were added as follows: ssDNA (39-mer, 130 ng), double-stranded DNA (dsDNA; 375 bp, 50 ng), pDNA (56 ng), or MacPCNA (12 pmol). The method used to make the pDNA is described above under “Electrophoretic Mobility Shift Assay.” Note that, for this purpose, the primer was not end-labeled. The sequences of the nucleic acids are shown in TABLE ONE. The thin layer chromatography plates were dried and exposed for autoradiography, and the results were quantified using a BAS-1800 II bio-imaging analyzer (Fuji Photo Film Co., Ltd.).

**A Unique Clamp Loader Complex in Archaea**

**Amino Acid Sequence Alignments**—All alignments were carried out with the multiple alignment program ClustalW (available at www.ebi.ac.uk/clustalw/), and the shading was manually carried out.

**RESULTS**

Two genes coding for two RFCS subunits and one coding for one RFCL subunit were identified in the genome sequence of *M. acetivorans* during genome annotation (available at www.broad.mit.edu/annotation/microbes/methanosarcina/). This finding was unlike those obtained for hitherto investigated archaeal genomes, which contain genes coding for one RFCS subunit and one RFCL subunit. To investigate whether this observation is unique to *M. acetivorans* or common to other members of the Methanosarcinaceae, we used the *M. acetivorans* genes to search for their orthologs in two other completely sequenced genomes from the genus *Methanosarcina*. In the genome sequences of *Methanosarcina mazei* (Mma) and *Methanosarcina barkeri* (Mba), the two RFCS genes were highly conserved. An alignment of the conserved motifs found in the gene products and those in their relatives is shown in Fig. 1. The Walker A motifs of MacRFCS2 and its orthologs in other members of the Methanosarcinaceae order deviate from the consensus sequence (GXXGXXGTK) of the archaeal and human RFCS proteins. The identities of the polypeptides for the RFCS subunits ranged from 80% (MacRFCS2 versus MbaRFCS2) to 97% (MacRFCS1 versus MmaRFCS1). In the case of the RFCL subunits, the identities ranged from 62% (MacRFCL versus MbaRFCL) to 75% (MacRFCL versus MmaRFCL). The conservation of the three genes in the three different species suggests either that members of the Methanosarcinaceae order have two clamp loader complexes made up of MacRFC-MacRFCS1 and MacRFC-MacRFCS2 or that one of the small subunits is nonfunctional, implying a single clamp loader complex composed of the large subunit and one of the two small subunits. Another interesting scenario is that these archaeal species harbor clamp loaders composed of three different proteins, as observed in bacteria. To determine which scenario leads to a functional clamp loader, we coexpressed the genes in the following combinations: rfcs1/rfcs1, rfcs1/rfcs2, and rfcs1/rfcs1/rfcs2. In addition, we expressed each gene individually to study the biochemistry of the gene product. The MacRFCS1 protein was made with and without an N-terminal His₆ tag; and in each case, the gene was highly expressed in *E. coli* cells. In contrast, the genes for MacRFCS2 and MacRFCL did not express unless with an N-terminal His₆ tag. The predicted molecular masses of MacRFCS1, MacRFCS2, and MacRFCL were 37,993, 38,385, and 67.5 kDa, respectively, and their predicted isoelectric points were 4.99, 4.88, and 8.53, respectively.

As shown in Fig. 2 (lanes 2–4), we succeeded in purifying each MacRFC subunit almost to homogeneity. It is of interest to note that the polypeptides of the methanosarchin RFCL subunit range from 602 amino acids (MbaRFCL) to 648 amino acids (MmaRFCL), whereas all other archaeal RFCL subunits in the GenBank™ Data Bank are 516 amino acids (*Methanococcus jannaschii* RFCL) or less. Upon coexpression of MacRFC and MacRFCS1, the two proteins coeluted in the void volume, suggesting either a very large product or an aggregated product lacking clamp loading activity (data not shown). Upon coexpression of MacRFC and MacRFCS2, although the two proteins were highly expressed in *E. coli* cells, we did not observe any coelution of the two proteins when antibodies were used for detection. However, a very stable complex was formed from the products of the three genes coexpressed together, and we purified this MacRFC complex by affinity, heparin, and size exclusion chromatographies. An aliquot of the peak elution fraction obtained by size exclusion chromatography was loaded in Fig. 2 (lane 5). Note that, in the recombinant MacRFC complex, MacRFCS2 and Mac-
RFCL were N-terminally His6-tagged to facilitate their expression. Mac-RFCS1 in the MacRFC complex was without the His6 tag sequence. We also observed a smaller protein band under RFCS1, and this protein coeluted consistently with the RFC complex in the gel filtration fractions (supplemental Fig. 1). Using a commercially available antibody against the His6 tag, we detected two proteins of ~67 and ~38 kDa by Western blot analysis (data not shown). The sizes of the two proteins corresponded well with those of His6-tagged MacRFCL and MacRFCS2, respectively. A polyclonal antibody raised against MacRFCS2 reacted with the ~38-kDa band, but not with the ~67-kDa band (data not shown). The presence of MacRFCS1 in the complex was detected by N-terminal sequencing. The band in Fig. 2 (lane 5) at ~35 kDa has an N-terminal amino acid sequence of MQALMEDS, which is an exact match for MacRFCS1. The N-terminal sequence of the band beneath MacRFCS1 is MKEEIIIE. We used this sequence to search the Escherichia coli genomes deposited in the GenBank Data Bank, and we did not find a perfect match. However, the sequence corresponded well with an internal sequence of MacRFCS1 (KEEIWIE). A protein harboring this sequence at the N terminus will lack the first 10 amino acids of MacRFCS1. Furthermore, polyclonal antibodies raised against MacRFCS1 reacted with both RFCS1 and the smaller band (data not shown). The protein may be either an aberrantly translated product or a truncated product. We intend to investigate this further in future experiments.

To determine the capacity of the MacRFC proteins to enhance DNA synthesis by MacPolBI in the presence of the cognate sliding clamp, we cloned and expressed the gene coding for a PCNA homolog in Methanothermobacter acetivorans (MacPCNA, GenBank accession number AAM03564). Following previous protocols (22), MacPCNA was purified to homogeneity, as shown in Fig. 2 (lane 6). In addition, we expressed the gene coding for MacPolBI and purified it (Fig. 2, lane 7) as described in our previous work (14). Gel filtration analysis was used to estimate the subunit organization of the MacRFC complex and its subunits in solution. Mac-RFCS1 eluted as a protein with a relative molecular mass of 72.4 ± 1.7 kDa.
Figure 3. ssDNA and pDNA binding activities of MacRFC subunits and the MacRFC complex. A, a fixed amount (0.5 pmol) of 32P-labeled ssDNA (lane 1) was incubated with increasing amounts (50, 100, 250, and 500 ng) of MacRFCL (lanes 2–5, respectively), MacRFCS1 (lanes 6–9, respectively), and MacRFCS2 (lanes 10–13, respectively). B, a fixed amount (0.5 pmol) of 32P-labeled ssDNA (lane 1) was incubated with increasing amounts (100, 250, 500, 750, 1000, 1500, and 2000 ng) of the MacRFC complex (lanes 2–8, respectively). C, a fixed amount (0.5 pmol) of 32P-labeled pDNA (lane 1) was incubated with increasing amounts (50, 100, 250, and 500 ng) of MacRFCL (lanes 2–5, respectively), MacRFCS1 (lanes 6–9, respectively), and MacRFCS2 (lanes 10–13, respectively). D, a fixed amount (0.5 pmol) of 32P-labeled pDNA (lane 1) was incubated with increasing amounts (100, 250, 500, 750, 1000, 1500, and 2000 ng) of the MacRFC complex (lanes 2–8, respectively). Each reaction mixture was incubated at 37 °C, and free DNA or the DNA-protein complex was resolved on 1% agarose gel. The results were then visualized by autoradiography.
12-fold by dsDNA, 58-fold by pDNA, and 3-fold by MacPCNA (Fig. 4B). The ATPase activity of MacRFC was further stimulated by addition of both nucleic acid and MacPCNA to the reaction mixture. However, addition of MacPCNA to MacRFC in the presence of pDNA did not yield higher ATP hydrolysis compared with pDNA alone as the effector. Interestingly, however, addition of both dsDNA and MacPCNA to the reaction mixture resulted in ATPase activity that was about four times that of MacRFC in the presence of only dsDNA. Mutation of the conserved lysine in the Walker A motif (RFC box III) (Fig. 1) to alanine resulted, in general, to decreases in the capacity of the MacRFC subunits to hydrolyze ATP (supplemental Fig. 2). Mutation of the conserved arginine in the SRC motifs (RFC box VII) (Fig. 1) of the RFCS subunits resulted in further drastic decreases in their capacity to hydrolyze ATP (supplemental Fig. 2, A and B). We also made MacRFC complexes harboring several combinations of mutations in the Walker A and SRC motifs. As observed for the MacRFCS subunits, mutations in the Walker A motif of individual subunits in the MacRFC complex led to decreases in ATP hydrolysis, with mutations in multiple subunits leading to further drastic decreases in ATPase activity (supplemental Fig. 3A). In this case also, mutant RFC complex proteins harboring arginine-to-alanine mutations in the SRC motif of either small subunit or both exhibited further drastic decreases in ATP hydrolysis (supplemental Fig. 3B).

In primer extension analysis, MacPolBI alone synthesized products of ~500 nucleotides in length (Fig. 5A, panels i–iv, lanes 2). Addition of only MacPCNA to MacPolBI failed to elicit an effect on the length of products synthesized by the DNA polymerase (Fig. 5A, panels i–iii, lanes 3). Addition of each of the MacRFC subunits to the reaction mixture in the presence of MacPCNA led to synthesis of shorter products by MacPolBI (Fig. 5A, panels i–iii, lanes 4). It is likely that MacPCNA increases the affinity of MacRFC subunits for ssDNA. The increase in protein binding to the M13mp18 ssDNA template then impeded synthesis by the DNA polymerase. In contrast, addition of MacRFC and MacPCNA to the reaction mixture stimulated primer extension, and full-length products (~7.2 kb) were synthesized (Fig. 5A, panel iv, lane 5). In the absence of MacPCNA, neither the MacRFC complex nor its subunits could stimulate an increase in primer extension by MacPolBI (Fig. 5A, panels i–iii, lanes 5). MacRFCS1 instead suppressed the length of the product synthesized (Fig. 5A, panel i, lane 5), which may reflect its higher affinity for ssDNA (Fig. 3A, lane 6). The results thus suggest that only the MacRFC complex has the capacity to enhance MacPCNA-dependent primer extension by MacPolBI.

We created several mutant MacRFC complexes harboring lysine-to-alanine mutations in the Walker A motif as described above. The first MacRFC mutant investigated was RFC-S1K/S2/L. This MacRFC mutant contained a single lysine-to-alanine mutation in MacRFCS1, and it exhibited appreciable ATPase activity in the presence of effectors,
although not as much as the wild-type MacRFC complex (supplemental Fig. 3A). As shown in Fig. 5B (panel i, lane 4), this mutant MacRFC complex failed to enhance MacPCNA-dependent primer extension by MacPolBI. The next mutant investigated was RFC-S1/S2K/L. This mutant MacRFC complex harbored a lysine-to-alanine mutation in only MacRFCS2. This mutant MacRFC complex maintained strong ATPase activity; and in the presence of some effectors, it even exhibited higher activity compared with the wild-type MacRFC complex (supplemental Fig. 3A). Interestingly, this mutant MacRFC complex was capable of stimulating MacPCNA-dependent primer extension by MacPolBI (Fig. 5B, lane 4). The next mutant MacRFC complex (designated RFC-S1/S2/LK) contained a single lysine-to-alanine mutation in only MacRFCL; and interestingly, this mutation led to further reduced ATPase activity compared with the wild-type MacRFC complex harboring a similar mutation in either MacRFCS1 or MacRFCS2 (supplemental Fig. 3A). As shown in Fig. 5B (panel iii, lane 4), this mutant MacRFC complex also stimulated MacPCNA-dependent primer extension by MacPolBI. The mutant MacRFC complexes containing the lysine-to-alanine mutations in both MacRFCS subunits (RFC-S1K/S2K/L) and also in all three subunits (RFC-S1K/S2K/LK) failed to stimulate primer extension by MacPolBI in the presence of MacPCNA (Fig. 5B, panels iv and v, lanes 4). These two mutants exhibited low ATPase activity and also failed, in general, to show any response to nucleic acids and MacPCNA in the ATP hydrolysis experiments described above.

Similar to other RFCL subunits described previously (5, 6), MacRFCL does not contain an SRC motif, unlike the MacRFCS subunits. Therefore, three different MacRFC mutants harboring mutations in the SRC motif of MacRFCS1 or MacRFCS2 or both (RFC-S1K/S2K/L, RFC-S1K/S2/L, and RFC-S1R/S2K/L) were made. Each of the three mutants exhibited very low ATPase activity even in the presence of nucleic acid and MacPCNA as effectors as described above. Whereas the mutant carrying the arginine-to-alanine mutation in only MacRFCS2 was able to enhance DNA synthesis by RFC-S1/S2/LK was not as efficient as that by the wild-type MacRFC complex (Fig. 5C, panels i–iii, lanes 5). As expected, the MacRFC complex with mutations in both RFCS subunits failed to stimulate primer extension by the DNA polymerase in the presence of MacPCNA (Fig. 5C, panel iv, lane 4).

The PfuRFC complex can be reconstituted by expressing the small subunit (PfuRFCS) and the large subunit (PfuRFCL) separately and then dialyzing excess amounts of PfuRFCS together with PfuRFCL (9). We investigated whether a functional MacRFC complex can be reconsti-
A Unique Clamp Loader Complex in Archaea

tuted by the same process. The mixtures tested were RFCS1/RFCS2, RFCL/RFCS1, RFCL/RFCS2, and RFCL/RFCS1/RFCS2. The products of RFCS1/RFCS2, RFCL/RFCS1, and RFCL/RFCS2 showed some increase in the length of the products synthesized by MacPolBI in the presence of MacPCNA (data not shown). Primer extension instead decreased in each case, which may be due to ssDNA binding by RFC subunits in the presence of PCNA and nucleotides, as described above. On the other hand, a similar experiment with the dialysate of RFCL/RFCS1/RFCS2 resulted in a polypeptide (MacPolBI) sequence at their C termini, as shown in Fig. 7B. Surprisingly, we found two PIP boxes, one that was likely to be the original PIP box in the methanosarcinal RFCL polypeptides, there appear to be two PIP boxes, one that was likely to be the original PIP box in the polypeptide (methanosarcinal PIP box I) (Fig. 6B) and another (methanosarcinal PIP box II) that was likely acquired together with the extended C terminus of the methanosarcinal RFCL subunit. The PIP boxes in the other archaeal organisms as stated above. Sequence analysis suggested the insertion of a peptide at the C-terminal end of the methanosarcinal RFCL subunit. As shown in Fig. 6B, most archaeal RFC proteins terminate with a peptide similar to the PCNA-interacting peptide (PIP) box (6). In the methanosarcinal RFC polypeptides, there appear to be two PIP boxes, one that was likely to be the original PIP box in the polypeptide (methanosarcinal PIP box I) (Fig. 6B) and another (methanosarcinal PIP box II) that was likely acquired together with the extended C terminus of the methanosarcinal RFCL subunit. The PIP boxes in the other archaeal organisms are the single highlighted PIP boxes found in the RFC subunits of these organisms (Fig. 6B).

We deduced that if the stimulation of primer extension by MacPolBI in the presence of MacPCNA and MacRFC is dependent on this structure for loading of the sliding clamp is dependent on this process. Among the three MacRFC subunits, only the E. coli cell line carrying all three genes produced a stable protein complex with the capacity to enhance MacPCNA-dependent primer extension by MacPolBI. Western blot analysis and N-terminal sequencing were used to confirm the presence of the three polypeptides in the recombinant MacRFC complex. In addition, the availability of polyclonal antibodies for the two small subunits allowed us to detect both proteins in M. aceticivorans cells (data not shown). Thus, the clamp loader complex of M. aceticivorans comprises two similar RFC subunits and one RFCI subunit. The genes coding for the three proteins are highly conserved in other members of the Methanobacterales order, including M. mazei, M. barkeri, and their psychrotolerant relative Methanococcales burtonii. Furthermore, genes encoding similar proteins were also found in the salt-loving archaeon Halobacterium sp. NRC-1. Thus, this hitherto unknown form of clamp loader may be more widely distributed in the archaeal domain than currently known.

The elution volume of the small subunit of PfuRFC suggests a protein that exists in solution as a homotetramer (9), and a similar subunit organization was also determined for the RFCS subunit of another hyperthermophile, Sulfolobus solfataricus (8). In the case of the Archaeoglobus fulgidus RFCS subunit, the protein eluted at a heterogeneous complex (26). Our gel filtration analysis (supplemental Fig. 1) resulted in a single symmetrical peak for each of the MacRFC subunits, and the elution volumes suggested a homodimer and a monomer for MacRFC1 and MacRFC2, respectively. Thus, the subunit organizations of the MacRFC subunits are very dissimilar to those of known archaeal RFCS subunits. MacRFC2 was expressed and purified as a His6-tagged protein, and although it eluted in the void volume, the highly purified protein (Fig. 2) exhibited both ATP hydrolysis and DNA binding activities. It is possible, however, that the aggregated RFC subunit might have occluded, from the host cells, proteins that contributed to its ATPase and DNA binding activities. The elution volume of the MacRFC complex suggested a protein with a relative molecular mass 341 kDa. The stoichiometry of the subunits in the MacRFC complex was not readily evident, and experiments aimed at obtaining this information are currently under way in our laboratory.

It has been reported that the A. fulgidus RFCS subunit does not bind to DNA significantly, whereas the RFCS subunit binds to DNA (26). The protein/DNA interaction of A. fulgidus RFC was therefore attributed mainly to the large subunit. In the PfuRFC proteins, however, the RFCS subunit clearly binds to pDNA, whereas the RFCL subunit shows only very weak pDNA binding activity (9). Interestingly, S. solfataricus RFC (8) does not bind to ssDNA, and Methanotrophus thermoautotrophicus (7) RFC binds to ssDNA only weakly. In contrast, the two proteins bind to ssDNA annealed with a primer (pDNA). These findings point to subtle differences in DNA binding activities in the archaeal RFC proteins and their subunits. A possible reason may be differences in the methods used in preparing the archaeal RFC subunits and also differences in the stability of these proteins in solution in the absence of their partners. However, it is clear that the archaeal functional clamp loaders bind to pDNA, the substrate onto which the clamp is loaded. Among the three MacRFC subunits, only MacRFC2 bound to pDNA, which suggests that the recognition of this structure for loading of the sliding clamp is dependent on this...
This finding is similar to that obtained with the human RFC subunits in that only the large subunit was demonstrated to bind to primer-template DNA (27). The effect of pDNA on the ATPase activities of the small subunits was not investigated because we did not observe binding of this substrate by the MacRFCS subunits. However, it must be noted that, in the presence of pDNA, the ATPase activities of the small subunits were not significantly altered. The mechanism by which this occurs remains to be determined.

The alignment of the amino acid sequences of regions differing in the archaeal two- and three-subunit clamp loaders is shown in Figure 6. The GenBank™ accession numbers of the proteins are as follows: MacRFCS1, AAM04110; MmaRFCS1, ZP_00561901; Halobacterium sp. NRC-1 RFCS (HspRFCS), ZP_00561901; M. burtonii RFCS1 (MbuRFCS1), ZP_00561901; M. thermoautotrophicus RFCS (MthRFCS), AAB84475; A. fulgidus RFCS (AfuRFCS), AAB84475; M. thermoautotrophicus RFCS (MthRFCS), AAB84475; and PfuRFCS, AAL82156. The PIP box sequences are shaded and denoted in the alignment. Conserved and similar amino acids are shaded black and gray, respectively. Note that PIP box II indicates the second PIP box in the methanosarcinal proteins. The other organisms have only one PIP box that aligns with methanosarcinal PIP box I.
of nucleotides, these proteins may bind to pDNA. Indeed, the ATPase activity of MacRFCS2, which showed only weak binding to ssDNA, was stimulated by nucleic acids, and this may suggest interaction with nucleic acids in the presence of ATP. The DNA binding activity of *A. fulgidus* RFC is enhanced by ATP (26). Similar to MacRFCL, PfuRFCL also elutes as a protein with a large relative molecular mass, but in contrast to MacRFCL, PfuRFCL exhibits extremely weak ATPase and DNA binding activities (9). The recombinant MacRFC complex possessed very weak ATPase activity, as reported for other archaeal RFC proteins (7–9, 26). However, the activity was highly stimulated by nucleic acids and MacPCNA. The preference of MacRFC for pDNA was evident, as its ATPase activity was most stimulated (58-fold) by this effector. This stimulation was higher than that reported for *M. thermoautotrophicus* RFC (4-fold) (7) and *S. solfataricus* RFC (10-fold) (8), but similar to that reported for *A. fulgidus* RFC (50-fold) (26).

To study the contribution of each of the RFC subunits to the integrity of the MacRFC complex as the clamp loader, we incorporated, into the RFC complex, different subunits carrying either a lysine-to-alanine mutation (as described above) in the Walker A motif or an arginine-to-alanine mutation in the SRC motif. All mutant RFC complex proteins

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**FIGURE 7.** A, alignment of the C-terminal regions of methanosarcinal and other archaeal PolBI proteins showing the two putative PIP boxes (I and II). MacPolBIΔC2 was truncated at the conserved glycine indicated with an asterisk; in the case of MacPolBIΔC1, only the C-terminal peptide (QRSLFDF) was deleted. The GenBank™ accession numbers of the proteins are as follows: MacPolBI, AAM04324; MmaPolBI, NP_634028; MbaPolBI, ZP_00543975; *Methanopyrus kandleri* PolBI (MkaPolBI), NP_614322; *M. thermoautotrophicus* PolBI (MthPolBI), AAB84714; PfuPolBI, AAL80336; *A. fulgidus* PolBI (AfuPolBI), NP_069333; and *S. solfataricus* PolBI (SsoPolBI), CAB57747. Conserved and similar amino acids are shaded black and gray, respectively. B, comparison of the primer extension activities of wild-type MacPolBI and putative PIP box-truncated derivatives. Lanes 1, template alone or negative control; lanes 2, template and truncated PolBI; lanes 3, template and wild-type MacPolBI; lanes 4, template, truncated MacPolBI, MacPCNA, and MacRFC; lanes 5, template, wild-type MacPolBI, MacPCNA, and MacRFC or positive control.
A Unique Clamp Loader Complex in Archaea

Acknowledgments—We thank Dr. Bryan A. White (University of Illinois at Urbana-Champaign) for discussions and Dr. William W. Metcalf (University of Illinois at Urbana-Champaign) for providing M. aceticivorum genomic DNA and also for insightful scientific discussions. Ernest K. D. Nyman and Claudia E. Guzman (Cann laboratory) are acknowledged for technical assistance.

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DECEMBER 23, 2005 • VOLUME 280 • NUMBER 51
JOURNAL OF BIOLOGICAL CHEMISTRY 41863

The Journal of Biological Chemistry (ISSN 0021-9258) is published weekly by The American Society for Biochemistry and Molecular Biology, Inc., 9650 Rockville Pike, Bethesda, MD 20814-3074. Annual subscription rates: USA and possessions, $9,426; all other countries, $9,426. For institutional subscription ordering information, contact Customer Service at 1-888-825-4242 or cs-jbc@am Soc.org. Second-class postage paid at Bethesda, MD, and at additional mailing offices. GST Reg. No. 124354488. Copyright © 2005 by the American Society for Biochemistry and Molecular Biology, Inc. All rights reserved. Periodicals postage paid at Bethesda, MD, and at additional mailing offices. POSTMASTER: Send address changes to The Journal of Biological Chemistry, Customer Service, 9650 Rockville Pike, Bethesda, MD 20814-3074.