Molecular Analyses of a Three-Subunit Euryarchaeal Clamp Loader Complex from Methanosarcina acetivorans†

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Chromosomal DNA replication is dependent on processive DNA synthesis. Across the three domains of life and in certain viruses, a toroidal sliding clamp confers processivity to replicative DNA polymerases by encircling the DNA and engaging the polymerase in protein/protein interactions. Sliding clamps are ring-shaped; therefore, they have cognate clamp loaders that open and load them onto DNA. Here we use biochemical and mutational analyses to study the structure/function of the Methanosarcina acetivorans clamp loader or replication factor C (RFC) homolog. M. acetivorans RFC (RFC$_{Ma}$), which represents an intermediate between the common archaeal RFC and the eukaryotic RFC, comprises two different small subunits (RFCS1 and RFCS2) and a large subunit (RFCL). Size exclusion chromatography suggested that RFCS1 exists in oligomeric states depending on protein concentration, while RFCS2 exists as a monomer. Protein complexes of RFCS1/RFCS2 formed in solution; however, they failed to stimulate DNA synthesis by a cognate DNA polymerase in the presence of its clamp. Determination of the subunit composition and previous mutational analysis allowed the prediction of the spatial distribution of subunits in this new member of the clamp loader family. Three RFCS1 subunits are flanked by an RFCS2 and an RFCL. The spatial distribution is, therefore, reminiscent of the minimal Escherichia coli clamp loader that exists in space as three γ-subunits (motor) flanked by the δ (stator) and the ϵ (wrench) subunits. Mutational analysis, however, suggested that the similarity between the two clamp loaders does not translate into the complete conservation of the functions of individual subunits within the RFC$_{Ma}$ complex.

Processive DNA synthesis is required to gain the speed necessary for the replication of large genomes as found in organisms across the three domains of life. Therefore, in each domain of life, molecular machines that ensure rapid DNA synthesis have evolved (17). At the center of the DNA synthesis machinery are the so-called replicative DNA polymerases and their processivity factors. Cells in all three domains of life, in addition to certain viruses, have evolved ring-shaped proteins, collectively called the processivity or sliding clamps, that repress the frequent dissociation of the DNA polymerase from the DNA template (17). The bacterial sliding clamp is known as the β-subunit, the eukaryotic functional homolog is called proliferating cell nuclear antigen (PCNA), and due to its similarity to the eukaryotic protein, the archaeal sliding clamp is also termed PCNA (4, 6, 12, 15). Among the viruses, the bacteriophage T4 clamp or gp45 has been well characterized (1). In each case, the clamp contains a cavity that is wide enough to allow it to encircle and slide freely along DNA (16, 18). However, since the clamp is a ring-shaped protein, it requires another protein to open and load it onto DNA. Thus, each clamp comes with its loader, also collectively known as the clamp loaders (2, 5, 29). Whereas the bacterial clamp loader is termed the γ-complex, the eukaryotic functional homolog is known as replication factor C or RFC (3, 19).

Cullmann and coworkers demonstrated that the heteropentameric RFC complex in eukaryotes is encoded by five different genes: four different genes coding for the RFC small subunit (RFCS) proteins and the last gene coding for the RFC large subunit (RFCL) proteins (9). In a subsequent analysis of clamp loaders in archaea, it was demonstrated that Methanothermobacter thermotrophicus (20), Sulfolobus solfataricus (29), and Pyrococcus furiosus (5) each contain only one RFCS gene and one RFCL gene. The polypeptide sequences of the RFCS of the archaea exhibit very high homology to those of the four eukaryotic RFCS (6), suggesting the relatedness of the archaeal/eukaryotic RFC proteins. Interestingly, four protomers of the archaeal RFCS protein form a complex with one large subunit protein to act as the functional clamp loader (5, 29). Thus, similar to the eukaryotic RFC, the archaea appeared to have a pentameric RFC complex that harnesses energy, derived from ATP hydrolysis, to break open the ring-shaped clamp. The loader then loads the opened clamp onto a primer-template junction, thereby facilitating the interaction between the clamp and its cognate replicative DNA polymerase to ensure processive DNA synthesis (10, 13, 14).

We recently described a new form of clamp loader in the domain Archaea (7). Unlike all previously described clamp loaders from this domain, the Methanosarcina acetivorans RFC

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(RFC₆₉₃) homolog has three different subunits instead of the usual two subunits, i.e., one small subunit and one large subunit. This new form of archaeal clamp loader may represent an intermediate stage in the evolution of the more complex RFC in eukaryotes from the less complex ones reported for archaia (7). In this report, we determined the subunit organization and propose the spatial distribution of subunits in the new form of the RFC complex. The predicted spatial distribution of the RFC₆₉₃ is similar to the Escherichia coli minimal γ-complex, which is arranged as the “stator,” “motor,” and “wrench.” Mutations in the predicted motor impaired the clamp loader’s capacity to stimulate clamp-dependent DNA synthesis by a cognate DNA polymerase in M. acetivorans (PolBI₆₉₃). C-terminal truncations that removed putative clamp interacting motifs in the subunit occupying the wrench position failed to abolish the capacity of the loader to stimulate clamp-dependent DNA synthesis by the cognate DNA polymerase. Our results, therefore, suggested that although the M. acetivorans and the E. coli clamp loaders likely adopt similar subunit organization/spatial distribution, the functions of the individual subunits are unlikely to be fully conserved.

### MATERIALS AND METHODS

**Cloning, expression, and purification of the M. acetivorans clamp loader complex.** The cloning, expression, and purification of RFC₆₉₃Ma, RFCCL₆₉₃Ma, and RFC₆₉₃ were described in our previous report (7). To create C-terminal truncation derivatives of RFCCL, a PCR method was used. All primers were shown in Table 1. To make five truncations of decreasing length from the C-terminal region of RFCCL₆₉₃Ma, PCR amplifications with a forward primer MacRFCCL in combination with five different reverse primers—RFCCL₁(1-475)R, RFCCL₃(1-432)R, RFCCL₄(1-410)R, and RFCCL₅(1-385)R—were carried out. The template was the gene coding for the wild-type RFCCL₆₉₃Ma, and the RFC complexes derived from the truncated proteins were designated RFCCL₁, RFCCL₂, RFCCL₃, RFCCL₄, and RFCCL₅, respectively. Each truncation targeted a region predicted to form a loop in the protein structure (Predictprotein [http://www.cbi.ac.uk/rrost/predictprotein/predictprotein.html]). Each PCR product was initially cloned into a vector Easy vector (Promega) and sequenced to confirm the integrity of the sequence. The RFCCL truncated genes were then individually cloned into pET28a as described for the wild-type RFCCL gene (7).

### Deletion of a 26-amino-acid insertion sequence in RFCCL₆₉₃Ma. A 26-amino-acid insertion sequence in RFCCL₆₉₃Ma was deleted by a PCR overlap method described in our previous report (30). The PCR primers that were used to delete the insertion sequence, as well as the flanking sequences, were MacRFCCL₂/F and RFC-L₆₉₃Ma/H9262 and MacRFCCL₄/F and RFC-L₆₉₃Ma/H9262 (Table 1).

### Point mutations in five RFC boxes in RFCCL₆₉₃Ma. The oligonucleotides that were used to create point mutations in RFC boxes II, IV, V, VI, and VIII of RFCCL are listed in Table 1 as RFCCL₁-K18A, RFCCL₁-E82A, RFCCL₁-E119A, RFCCL₁-E136A, and RFCCL₁-D205A. The method used was as described by the manufacturer for the QuikChange site-directed mutagenesis kit (Stratagene). All DNA inserts were sequenced (W. M. Keck Center for Comparative and Functional Genomics, University of Illinois at Urbana-Champaign) to confirm the presence of the desired mutations.

### Estimation of subunit organization by size exclusion chromatography. To estimate the subunit organization of individual RFC₆₉₃Ma subunits and also their mixtures, the purified proteins were dialyzed against buffer A containing 50 mM Tris·HCl (pH 8.0) and 300 mM NaCl. Different concentrations of each protein in a 100-μl total volume were then loaded onto a Superdex 200 HR 10/30 gel filtration column (GE Healthcare) already equilibrated with the same buffer, and the chromatography was developed at 4°C. For the mixtures of RFC₆₉₃Ma, RFCCL₆₉₃Ma, and RFC₆₉₃, various amounts of the individual proteins were mixed and dialyzed against buffer A for 1 h before the gel filtration analysis. The chromatography was developed with the same buffer at a flow rate of 0.5 ml/min, and fractions of 0.5 ml were collected and aliquots were analyzed on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The gel filtration 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 RNase A, 13.7 kDa) under the same conditions.

### TABLE 1. Oligonucleotides used in this study

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<tr>
<th>Experiment</th>
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<th>Nucleotide sequence</th>
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<td></td>
<td>MacRFCCL2R</td>
<td>TTTTCTGTCAGGAGGTATAGTGGAATTCAGG</td>
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<td></td>
<td>RFCCL3(1-410)R</td>
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<td></td>
<td>RFCCL3(1-385)R</td>
<td>CTTCGAGTTATTTGCCGGCAGAACCTTCCTTTTG</td>
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<td></td>
<td>RFCCL1-E82A</td>
<td>AGGGAACATTTTTCGACATTGCTTCCGAT</td>
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<td></td>
<td>RFCCL1-E119A</td>
<td>ATCATTTTTCTGTAGCAGGGCTGTTACAA</td>
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<td></td>
<td>RFCCL1-E136A</td>
<td>CTCCGAGACCAGCTACGCGTTCAGCACGACAC</td>
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<td></td>
<td>RFCCL1-D205A</td>
<td>TACGATAGCTTACGAGGAGCTCATCGGAAAACTGTC</td>
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<td>Primer extension</td>
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<td>ATTCGTAATCATGGTCAAGTTTCTTCTTG</td>
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*The experiments in which the oligonucleotides were used are indicated. The truncation oligonucleotides were used to truncate RFCCL₆₉₃Ma and RFCCL₆₉₃Ma. The mutation oligonucleotides were used to create site-directed mutations in the RFC boxes of the RFCCL₆₉₃Ma gene. The primer extension oligonucleotide was used in DNA synthesis with M13 ssDNA as the template. Restriction sites incorporated into the oligonucleotides are underlined.*
Stoichiometry of RFC<sub>Tm</sub> complex and spatial arrangement. The stoichiometric ratio of RFC<sub>S1</sub>, RFC<sub>S2</sub>, and RFCL in the RFC<sub>Tm</sub> complex was determined through a densitometric method as in our previous report on Pyrococcus furiosus RFC (RFC<sub>Pf</sub>) complex (5). The RFC<sub>Tm</sub> complex was resolved through SDS-PAGE, and after being stained with Coomassie brilliant blue followed by destaining, the gels were scanned with a densitometer. Each band was then quantified from a calibration curve obtained by using each purified subunit at different concentrations to obtain a relationship between protein band intensity and the amount of protein loaded for the electrophoretic analysis.

CD scan. The wild-type RFC<sub>Tm</sub> complex and its mutants were subjected to circular dichroism (CD) scans to determine if individual mutations impacted the secondary structural elements of the protein complex. The method used was the same as described in our previous report (23). However, in this case, we used 0.5 μg/μl of each protein in a buffer composed of 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5 mM MgCl<sub>2</sub>, 10% glycerol, and 1 mM diithiothreitol. Triplicate data sets were collected per protein under investigation, and all data were normalized against readings obtained from buffer without protein.

Cloning, expression, and purification of PCNA<sub>Tm</sub> and PolB<sub>Tm</sub>. The methods for the production of <i>M. acetivorans</i> PCNA or the sliding clamp and the <i>M. acetivorans</i> DNA PolB<sub>Tm</sub> were as described in our previous report (7).

Primers for <i>M. acetivorans</i> DNA PolB<sub>Tm</sub> were designed based on the PCNA trimer and are complementary to positions 6205 to 6234 of the template, were annealed by heating to 95°C for 5 min and cooling slowly to room temperature in a buffer composed of 20 mM Tris-HCl (pH 8.8), 100 mM NaCl, 5 mM MgCl<sub>2</sub>, and 2 mM β-mercaptoethanol. Where indicated, restriction enzymes were added to the annealing reaction mixture, which was scaled up by the number of reactions required. Each primer extension reaction (total volume of 20 μl) contained 1 pmol of the end-labeled primer annealed to 500 ng of template, 250 μM of each deoxynucleoside triphosphate, and 20 mM ATP in the buffer used for the annealing reaction. To initiate primer extension, the cognate DNA polymerase, PolB<sub>Tm</sub>, was added to the reaction mixture at 0.24 μM. Where the effects of accessory factors were investigated, they were added at 77.5 nM of RFC<sub>Tm</sub> complex or its mutants, and 0.3 μM of PCNA<sub>Tm</sub> (based on the PCNA trimer) per reaction. In the case of the RFC<sub>Tm</sub>, the amounts of RFC<sub>Tm</sub> added ranged from 1.5 to 15 μM/μl/reaction. The primer extension reaction was carried out at 37°C for 5 min and terminated with 6 μl of stop solution (98% formamide, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue). After being heated at 95°C for 5 min, the samples were resolved on a 1% alkaline agarose gel and visualized by autoradiography.

Amino acid sequence alignments. The amino acid sequence alignments were carried out using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and the shading was manually carried out.

RESULTS

Oligomerization state of <i>M. acetivorans</i> clamp loader small subunits. We successfully expressed both RFC<sub>S1<sub>Tm</sub></sub>, RFC<sub>S2<sub>Tm</sub></sub>, and RFCL in the functional RFC complex was determined through a densitometric method as described in our previous report for the Pyrococcus furiosus RFC complex (5). The concentrations of the RFC subunits shown in Fig. 2A were calculated to be 71.0 pmol, 25.6 pmol, and 22.4 pmol for RFCS1<sub>Tm</sub>, RFCS2<sub>Tm</sub>, and RFCL<sub>Tm</sub>, respectively. By mixing RFCS1<sub>Tm</sub>, RFCS2<sub>Tm</sub>, and RFCL<sub>Tm</sub> at concentrations of 20/2, 20/5, and 20/20 μg/μl, respectively, we observed two peaks representing dimers (82.0 ± 0.9 kDa) and monomers (37.4 ± 0.1 kDa). This suggested that the two proteins interacted and that the dimers were likely heterodimers of the two proteins. RFCS1<sub>Tm</sub> at 5 μg/μl eluted as a dimer (Fig. 1A). However, when mixed with RFCS2<sub>Tm</sub> at 20 μg/μl, there were two peaks that were estimated as 104.1 ± 8.0 kDa and 38.2 ± 0.6 kDa. This may suggest a heterotrimer of RFC<sub>S1</sub><sub>Tm</sub>/RFC<sub>S2</sub><sub>Tm</sub>/monomers of the individual proteins, respectively. By mixing RFC1<sub>Tm</sub>/RFC2<sub>Tm</sub>, and RFCL<sub>Tm</sub> at concentrations of 20/2, 20/5, and 20/20 μg/μl, respectively, we observed two peaks. The elution volume of the first peak suggested either a trimer or tetramers of the RFC proteins (117 to 127 kDa), and the second peak suggested monomers (36.2 to 38.2 kDa).

Subunit organization and spatial distribution of the RFC<sub>Tm</sub> complex. The stoichiometric ratio of RFC<sub>S1</sub>, RFC<sub>S2</sub>, and RFCL in the functional RFC complex was determined through a densitometric method as described in our previous report for the Pyrococcus furiosus RFC complex (5). The concentrations of the RFC subunits shown in Fig. 2A were calculated to be 71.0 pmol, 25.6 pmol, and 22.4 pmol for RFC1<sub>Tm</sub>, RFC2<sub>Tm</sub>, and RFCL<sub>Tm</sub>, respectively. In the absence of RFC1<sub>Tm</sub>, RFC2<sub>Tm</sub> eluted at a peak elution volume of 14.8 ml (Fig. 1B). However, when the two purified proteins were dialyzed together and analyzed by size exclusion chromatography, RFC2<sub>Tm</sub> eluted earlier with RFC1<sub>Tm</sub>, suggesting an interaction between the two proteins (Fig. 1C). RFC1<sub>Tm</sub> alone at 2 μg/μl was estimated as a monomer (Fig. 1A), and at a concentration of 20 μg/μl, RFC2<sub>Tm</sub> also eluted as a monomer (Fig. 1B). However, when mixed RFC1<sub>Tm</sub> and RFC2<sub>Tm</sub> at 2 μg/μl and 20 μg/μl, respectively, we observed two peaks representing dimers (82.0 ± 0.9 kDa) and monomers (37.4 ± 0.1 kDa). This suggested that the two proteins interacted and that the dimers were likely heterodimers of the two proteins. RFC1<sub>Tm</sub> at 5 μg/μl eluted as a dimer (Fig. 1A). However, when mixed with RFC2<sub>Tm</sub> at 20 μg/μl, there were two peaks that were estimated as 104.1 ± 8.0 kDa and 38.2 ± 0.6 kDa. This may suggest a heterotrimer of RFC1<sub>Tm</sub>/RFC2<sub>Tm</sub>/monomers of the individual proteins, respectively.
The PIP boxes are known to mediate interactions with sliding clamps or PCNA (34). We, therefore, carried out a series of truncations from the C terminus of RFC<sub>Ma</sub> to determine whether the removal of elements in the C-terminal region, especially the PIP boxes, will affect clamp stimulation of DNA synthesis in the presence of the RFC<sub>Ma</sub> complex. As shown in Fig. 3B, we successfully produced four of the five RFC complex proteins that contained truncated derivatives of RFC<sub>L5</sub>Ma. The last RFC<sub>L5</sub>-truncated derivative, RFC-L<sub>5</sub>A (which has the last 222 amino acids removed), could not be obtained (results not shown). An examination of the E. coli cells expressing this RFC complex showed that the truncated RFC failed to express. The first and second truncation mutants, RFC-L<sub>1</sub>A and RFC-L<sub>2</sub>A, removed PIP box 1 and both PIP box 1/PIP box 2, respectively (7). However, as shown in Fig. 3D, lanes 4, both mutant clamp loader complexes stimulated PCNA<sub>Ma</sub>-dependent DNA synthesis by PolB<sub>Ma</sub>, suggesting that both RFC-L<sub>1</sub>A and RFC-L<sub>2</sub>A are capable of loading the sliding clamp. The results were, therefore, similar to those of the primer extension in the presence of the wild-type RFC complex (Fig. 3D, lanes 6). The subsequent deletions that removed 175 amino acids (RFC-L<sub>3</sub>Ma) and 197 amino acids (RFC-L<sub>4</sub>A; Fig. 3C) from the C terminus, respectively, also did not lead to the failure to load PCNA<sub>Ma</sub> (Fig. 3D, lanes 4).

Mutations in some conserved RFC boxes of RFC<sub>1Ma</sub> impair RFC<sub>Ma</sub>-dependent PCNA<sub>Ma</sub> stimulation of DNA synthesis by PolB<sub>Ma</sub>. Eight conserved motifs, referred to as RFC box I to box VIII, were previously identified in RFC proteins (9). The conservation of seven of these RFC boxes (box II to box VIII) was later confirmed in archaeal RFC proteins (6). In the present work, we hypothesized that other than RFC box III (Walker A motif or P-loop) and box VII (SRC motif), which have been shown to be critical for ATPase activity and, therefore, the clamp loading function of RFC proteins including the RFC<sub>Ma</sub> complex (7), the other RFC boxes, especially those in the putative motor domain, are likely to play important roles in the function of this new clamp loader. To test our hypothesis, we made a single mutation at a conserved residue in each of RFC box II [RFC<sub>1Ma</sub>(K18A)], box IV [RFC<sub>1Ma</sub>(E82A)], box V [RFC<sub>1Ma</sub>(E119A)], box VI (D136A), and box VIII (D205A) (Fig. 4). Each mutant RFC<sub>1Ma</sub> was used in reconstituting an RFC<sub>Ma</sub> complex that harbored that single mutation. To ensure that each mutation did not lead to gross changes in the secondary structural elements, each RFC complex containing the respective mutation was highly purified (results not shown) and subjected to CD scan. As shown in Fig. 5A, except for the RFC complex protein containing RFC<sub>1Ma</sub>-E119A (mutation in box V), the other mutant RFC complex proteins exhibited CD spectra that were similar to that of the wild-type RFC<sub>Ma</sub> complex. It should be noted, however, that the clamp interaction (17). In Fig. 3A, the M. acetivorans RFC<sub>L5</sub>, which occupies the position of the wrench in the E. coli clamp loader, has two putative PCNA interacting protein (PIP) boxes at the C-terminal region: PIP box 1 (QKTLFDF; amino acids 596 to 602) and PIP box 2 (QKTLNMGF; amino acids 488 to 495) in Fig. 3C. The PIP boxes are known to mediate interactions with sliding clamps or PCNA (34).
FIG. 3. Hypothetical functions of the M. acetivorans clamp loader subunits. (A) The proposed model of the RFC<sub>Ma</sub> subunit arrangement is similar to that of the E. coli minimal clamp loader, the γ-complex. The RFC<sub>S2Ma</sub>, RFC<sub>S1Ma</sub>, and RFC<sub>LMa</sub> subunits occupy the positions of the stator, the motor, and the wrench, respectively, in the E. coli protein complex. (B) SDS-PAGE of the purified recombinant wild-type RFC<sub>Ma</sub> complex and RFC<sub>Ma</sub> complex proteins containing truncated RFC<sub>LMa</sub>. Lane 1, protein molecular mass markers (Fermentas); lane 2, wild-type RFC<sub>Ma</sub> complex; lane 3, M. acetivorans RFC-L<sub>H9004</sub>1 complex; lane 4, M. acetivorans RFC-L<sub>H9004</sub>2 complex; lane 5, M. acetivorans RFC-L<sub>H9004</sub>3 complex; lane 6, M. acetivorans RFC-L<sub>H9004</sub>4 complex. (C) Alignment of the C-terminal region of RFCL<sub>Pf</sub> (PfuRFCL) and RFCL<sub>Ma</sub> (MacRFCL) showing the single and double PIP boxes in RFCL<sub>Pf</sub> and RFCL<sub>Ma</sub>, respectively. In addition, the points of the truncations in RFCL<sub>Ma</sub> are denoted by asterisks and the different truncations shown as L<sub>H9004</sub>X, where X represents 1, 2, 3, 4, or 5. The basic amino acid sequence in RFCL<sub>Ma</sub> (KEKEKPKKGK) and the mostly acidic amino acid sequence (EEEKEEEEEE KPEEEKEEE) are shown. (D) Effects of RFC<sub>Ma</sub> complex proteins containing truncated RFC<sub>LMa</sub> on the primer extension capacity of PolBI<sub>Ma</sub> in the presence of PCNA<sub>Ma</sub>. Primer extensions were compared as follows: lanes 1, template alone or negative control; lanes 2, template/PoBI<sub>Ma</sub>; lanes 3, template/PoBI<sub>Ma</sub>/PCNA<sub>Ma</sub>; lanes 4, template/PoBI<sub>Ma</sub>/PCNA<sub>Ma</sub>/M. acetivorans RFC-LΔ; lanes 5, template/PoBI<sub>Ma</sub>/M. acetivorans RFC-LΔ mutants; lanes 6, template/PoBI<sub>Ma</sub>/PCNA<sub>Ma</sub>/RFC<sub>Ma</sub> wild type or positive control. The substrate for DNA synthesis was 0.5 μg of singly primed M13mp18 ssDNA in DNA polymerase reaction buffer (described in Materials and Methods). The reaction mixtures were incubated at 37°C for 5 min, and the products were resolved by 1% alkaline agarose gel electrophoresis and visualized by autoradiography. Note that the RFC<sub>Ma</sub> complex in the absence of PCNA has no effect on DNA synthesis by PolBI<sub>Ma</sub> (7).
FIG. 4. An alignment showing the conserved RFC boxes in RFCS in both archaea and eukaryote Saccharomyces cerevisiae. The GenBank accession numbers of the proteins are as follows: Methanosarcina acetivorans C2A RFCS1 (MacRFCS1), NP_615630; Methanocaldus marisnigri JRI1 RFCS1 (MmariRFCS1), YP_001047161; Methanospirillum hungatei JF-1 RFCS1 (MhunRFCS1), YP_502463; Halobacterium sp. strain NRC-1 RFCS1 (HsRFCS1), NP_280914; Haloarcula marismortui RFCS1 (HmaRFCS1), YP_137064; Haloquadratum walsbyi DSM RFCS1 (HwaRFCS1), YP_659332; Archaeoglobulus fulgidus RFCS (AfuRFCS), NP_070884; Pyrococcus furiosus RFCS (PfuRFCS), NP_577822; Methanothermobacter thermautotrophicus RFCS (MthRFCS), NP_275384; Sulfolobus solfataricus RFCS (SsoRFCS), NP_342275; Natronomonas pharaonis RFCS1 (NapRFCS1), YP_326110; Saccharomyces cerevisiae RFCS3 (SceRFC3p), NP_014109; S. cerevisiae RFCS2 (SceRFC2p), EDV10522; S. cerevisiae RFCS5 (SceRFC5p), NP_0109644. Organisms with subunits designated -S1 are archaeal organisms with two RFCS (refer to the legend to Fig. 6). Conserved and similar amino acid residues are shaded black and gray, respectively. The asterisks denote the residue mutated to alanine in a given RFC box. Mutations in the RFC boxes with arrowheads impaired RFC-dependent PCNA<sub>Ma</sub> stimulation of DNA synthesis by PolB<sub>Ma</sub>.

The clamp loader complex containing E82A (RFC box IV) also showed some deviation from the wild type. The results, thus, suggested that at least three of the mutations (K18A, E136A, and D205A) did not severely impact the secondary structural elements of the RFC<sub>Ma</sub> complex. Using primer extension analysis, we observed that three of the mutations made in RFCS1<sub>Ma</sub> (putative motor) impaired the capacity of the mutant RFC complex to stimulate PCNA<sub>Ma</sub>-dependent DNA synthesis by PolB<sub>Ma</sub>. These were the mutations in RFC box IV (E82A; Fig. 5Bii, lane 4), box V (E136A; Fig. 5Biv, lane 4), and box VIII (D205; Fig. 5Bv, lane 4). While the mutation in RFC box II (K18A) did not appear to impair the capacity of the RFC<sub>Ma</sub> complex to stimulate PCNA<sub>Ma</sub>-dependent DNA synthesis by PolB<sub>Ma</sub> (Fig. 5Bii, lane 4), we observed a decreased aptitude in the case of the clamp loader complex containing the E119A mutation (Fig. 5Biii, lane 4). Each protein was compared with the wild-type RFC<sub>Ma</sub> complex, serving as a positive control (Fig. 5B, lanes 6).

The insertion sequence downstream of RFC box IV in RFCS2<sub>Ma</sub> is not required for clamp loading. In archaeal RFCS2 polypeptides deposited in GenBank (http://www.ncbi.nlm.nih.gov/), an insertion sequence occurs downstream of RFC box IV (9) as shown in Fig. 6A. In the order Methanosarcinales, the insertion sequence comprises 26 amino acids that are highly conserved (7). To determine if the insertion sequence plays a role in PCNA loading, a PCR approach was used to delete the insertion sequence from RFCS2<sub>Ma</sub>, and an RFC complex containing the mutant RFCS2<sub>Ma</sub> instead of the wild-type protein, was highly purified (results not shown). The RFC<sub>Ma</sub> complex containing the mutant RFCS2 (RFCS2; Fig. 6B) was tested for its capacity to enhance primer extension by PolB<sub>Ma</sub> in the presence of the cognate clamp or PCNA<sub>Ma</sub>. As shown in Fig. 6B, lane 4, deletion of the insertion sequence in RFCS2<sub>Ma</sub>, although it appears to have slightly impaired the capacity of the protein complex to stimulate PCNA<sub>Ma</sub>-dependent DNA synthesis by PolB<sub>Ma</sub>, did not abolish the clamp loading activity. We look forward to characterizing the mutant in detail. It should also be noted that the deleted sequence may play a role in a different function, for example, in interactions with other proteins.

The RFCS<sub>Ma</sub>/RFCS<sub>2Ma</sub> complex is unable to stimulate PCNA<sub>Ma</sub>-dependent DNA synthesis by PolB<sub>Ma</sub>. As demonstrated above, we detected interactions that suggested heterodimer formation and either heterotrimer or heterotetramer formation between RFCS<sub>1Ma</sub> and RFCS<sub>2Ma</sub> (Fig. 1C). The peak fraction from each subunit organization was used, in a primer extension analysis, to determine whether the complex of the M. acetivorans RFCS can stimulate PCNA<sub>Ma</sub>-dependent DNA synthesis by PolB<sub>Ma</sub>. In each case, the complex of the small subunits rather inhibited DNA synthesis, suggesting that they cannot load PCNA (see Fig. S1 in the supplemental material). The results were, therefore, similar to our previous findings that the individual subunits of RFC inhibit DNA synthesis by PolB<sub>Ma</sub> (7). A similar finding showing the inhibition of a cognate DNA polymerase (Pol 6) by human RFCS has been reported elsewhere (28).

**DISCUSSION**

*Pyrococcus furiosus* RFCS (5, 27) and *Archaeoglobus fulgidus* RFCS (32) were shown to exist in oligomeric forms. Unlike the usual archaean clamp loader proteins, the RFC<sub>Ma</sub> complex has
FIG. 5. Mutational analysis within different conserved RFC boxes in the motor domain of the RFC<sub>E<sub>Ma</sub></sub> complex. (A) The highly purified RFC<sub>E<sub>Ma</sub></sub> complex and its RFC box mutant derivatives were subjected to CD scans. The CD spectra of each protein from 200 nm to 260 nm were collected in triplicate and normalized against readings from buffer without protein. The samples analyzed were the wild-type RFC<sub>E<sub>Ma</sub></sub> complex (MacRFC-WT); RFCS1(K18A)<sub>E<sub>Ma</sub></sub> complex [MacRFC-S1(K18A)], mutation in RFC box II; RFCS1(E82A)<sub>E<sub>Ma</sub></sub> complex [MacRFC-S1(E82A)], mutation in RFC box IV; RFCS1(E119A)<sub>E<sub>Ma</sub></sub> complex [MacRFC-S1(E119A)], mutation in RFC box V; RFCS1(E136A)<sub>E<sub>Ma</sub></sub> complex [MacRFC-S1(E136A)], mutation in RFC box VI; and RFCS1(D205A)<sub>E<sub>Ma</sub></sub> complex [MacRFC-S1(D205A)], mutation in RFC box VIII. (B) Effect of individual mutations in the RFC boxes on the capacity of the RFC<sub>E<sub>Ma</sub></sub> complex to stimulate primer extension of PolBI<sub>E<sub>Ma</sub></sub> in the presence of PCNA<sub>E<sub>Ma</sub></sub>. Lane 1, template
two small subunits (7). In the present report, we show that RFCS1$_{Ma}$, can exist as monomers and also as oligomers, ranging from homodimers to homotetramers, and that the oligomerization state is dependent on the protein concentration. In contrast, RFCS2$_{Ma}$ only exists as monomers in solution, irrespective of the protein concentration. The amino acid sequence alignment of RFCS1$_{Ma}$ and RFCS2$_{Ma}$ with other archaeal RFCS proteins (results not shown) suggests that RFCS1$_{Ma}$ is more similar to the known archaeal RFCS proteins. Thus, the RFCS1$_{Ma}$ polypeptide exhibits 59%, 50%, 49%, and 63% identities to the RFCS proteins found, respectively, in _P. furiosus_, _Methanothermobacter thermautotrophicus_, _Sulfobolus solfatarius_, and _A. fulgidus_ (5, 20, 29, 31). RFCS2$_{Ma}$, on the other hand shows 29 to 38% identities to these proteins. A mixture of RFCS1$_{Ma}$/RFCS2$_{Ma}$ coeluted during size exclusion chromatography (Fig. 1C), suggesting that the two proteins interacted in solution. Although the protein complexes are likely to contain both RFCS1$_{Ma}$ and RFCS2$_{Ma}$, they may also contain protein complexes made up of only RFCS1, since this protein can undergo self-oligomerization. Samples from the peak fractions of the oligomerized RFCS1$_{Ma}$/RFCS2$_{Ma}$ failed to stimulate PCNA$_{Ma}$-dependent DNA synthesis by PolBl$_{Ma}$, suggesting that the complexes of the RFCS$_{Ma}$ proteins are incapable of loading the sliding clamp.

The stoichiometry and spatial distribution of RFCS$_{Ma}$ subunits in the functional clamp loader were also determined in this report. In our model, three RFCS$_{Ma}$ protomers are flanked by an RFCS2$_{Ma}$ on one side and by an RFCL$_{Ma}$ on the other side (Fig. 2C). This arrangement can be inferred from the estimated stoichiometry and our previous mutational analysis that showed that a critical mutation in the Walker A motif of RFCS2 has no effect on clamp-loading activity (7). The observation implied that the Walker A motif of RFCS2$_{Ma}$ does not participate in ATP binding/hydrolysis. RFC proteins are AAA$^+$ ATPases (17), and the ATPase sites are formed by a Walker A motif (RFC box III) from one subunit and an arginine finger (R in the SRC motif or RFC box VII) from an adjacent subunit. In the spatial arrangement shown in Fig. 2C, the Walker A (P-loop) of RFCS2 does not participate in ATP binding, and therefore, it does not influence ATP hydrolysis. A mutation in the Walker A motif of RFCL$_{Ma}$ (one ATPase site) also did not impair clamp-loading activity by RFCL$_{Ma}$. On the other hand, mutations in either the SRC or Walker A motif of RFCS1$_{Ma}$, which should affect three ATP binding sites according to our predicted spatial distribution (Fig. 2C), abolished clamp-loading activity (7). Both ATP binding and hydrolysis are required for the conformational changes that lead to clamp loading (17), and our previous mutational data (7) therefore support our proposed spatial distribution. The spatial arrangement of the RFCS$_{Ma}$ complex is, therefore, likely to be similar to the _E. coli_ minimal clamp loader (10, 17).

Subunits of the _E. coli_ q-clamp are capable of binding to the sliding clamp or $\beta$-subunit. The $\delta$ wing exhibits the strongest interaction with the $\beta$-clamp, and by itself, it can load and unload the clamp (17, 21, 33). The $\gamma$, on the other hand, binds less tightly and is also less competent in unloading the $\beta$-clamp (22). The $\delta'$ is known as the stator or the stationary part of the $\gamma$-complex upon which the other subunits move, and its arginine finger together with the P-loop or Walker A motif of the adjacent $\gamma$-protomer forms the first ATPase site (site 1) in the $\gamma$-complex (35), as also shown in Fig. 3A for RFC$_{Ma}$. In archaea, RFCL is known to interact with the sliding clamp or PCNA (4). The C-terminal region of RFCL$_{Ma}$, which occupies the position analogous to the _E. coli_ wrench, harbors two putative PIP boxes (Fig. 3C), although previously described RFCL proteins in archaea have only one PIP box (4, 6). This interaction between PCNA and the box by analogy, is similar to the interaction between the wrench (clamp-opening subunit) and the sliding clamp. Each of our four deletions from the C terminus of RFCL$_{Ma}$ (Fig. 3B), however, failed to abolish the stimulation of PCNA-dependent enhancement of DNA synthesis by PolBl$_{Ma}$. Thus, it is likely that although the subunit organization and spatial distribution of the RFC$_{Ma}$ complex are similar to the minimal _E. coli_ clamp loader, interaction through the PIP boxes in the large subunit (wrench position) is not required for clamp loading. Note that in _Methanosarcina acetivorans_, instead of an octapeptide PIP box, several PCNA-interacting proteins have rather a conserved heptapeptide PIP box (7; I. K. O. Cann et al., unpublished data).

It was demonstrated previously that the RFCL$_{py}$ contains 10 amino acids that form a basic cluster (motif) immediately upstream of the PIP boxes (26). These clusters are present in RFCL$_{Ma}$ (Fig. 3C). However, unlike the RFCL$_{py}$ and its homologs in other thermophilic archaea, such as _Aeropyrum pernix_, _Methanocaldococcus jannaschii_, and _M. thermautotrophicus_, the RFCL$_{Ma}$ has $>$30 amino acids separating the basic residues from the most C-terminal PIP box. Furthermore, the acidic-residue-rich (glutamic acid-rich) region is split by several amino acids in the case of RFCL$_{Ma}$ (Fig. 3C). The basic amino acid cluster, and not the acidic cluster, was shown to be required for the formation of a ternary complex of RFC$_{py}$/PCNA$_{py}$/DNA (26). Furthermore, although the PIP box in RFCL$_{py}$ was shown to bind tightly to the sliding clamp (24), in support of our data presented here, the PIP box was shown to be nonessential for the RFC-dependent enhancement of DNA synthesis by PolBl$_{py}$ in the presence of its cognate PCNA (24).

The analysis of the polypeptides constituting the eukaryotic RFC proteins clearly showed that they contain conserved motifs that were designated RFC boxes (9). These motifs were also conserved in archaeal RFC proteins (6, 7). Although RFC box I is unique to the eukaryotic RFCL, box II to box VIII are common to all RFCS proteins (6). The functions of RFC box III (Walker A motif) and RFC box VII (SRC motif) in the...
archaeal and eukaryotic proteins are well described since the two motifs fold to form the competent ATPase sites in the protein complex (3, 10). To our knowledge, the other RFC boxes have not been investigated for their contribution to the structure/function of an RFC complex. Thus, as a first step to understanding the importance of these motifs in the RFC complex, we targeted conserved amino acids in the RFC boxes of RFCS1Ma, which may serve as the motor in the clamp loader complex. The importance of RFCS1 was demonstrated in our previous report, where we showed that single mutations in a conserved lysine in the Walker A motif or the conserved arginine in the SRC motif in this subunit abolished RFC-dependent stimulation of PolBI Ma during DNA synthesis in the presence of PCNA. This observation suggested failure to load the clamp by the mutant RFC Ma complex (7). Interestingly, E119A slightly stimulated PCNA Ma-dependent DNA synthesis by PolBI Ma, which may serve as the motor in the clamp loader complex. The importance of RFCS1 was demonstrated in our previous report, where we showed that single mutations in a conserved lysine in the Walker A motif or the conserved arginine in the SRC motif in this subunit abolished RFC-dependent stimulation of PolBI Ma during DNA synthesis in the presence of PCNA. This observation suggested failure to load the clamp by the mutant RFC Ma complex (7). Interestingly, E119A slightly stimulated PCNA Ma-dependent DNA synthesis by

FIG. 6. Effect of an insertion sequence in RFCS2Ma on PCNA Ma-dependent DNA synthesis by PolBI Ma. (A) Alignment depicting insertion sequences downstream of RFC box IV regions in RFCS2Ma and its homologs. The GenBank accession numbers of the proteins are as follows: M. acetivorans RFCS1 (MacRFCS1), NP_615630; M. marmoreus RFCS1 (MmarisRFCS1), YP_137064; N. pharaonis RFCS1 (NphaRFCS1), YP_326110; Halobacterium sp. strain NRC-1 RFCS1 (HspRFCS1), NP_280914; Haloarcula marismortui RFCS1 (HmarisRFCS1), YP_136998; N. pharaonis RFCS2 (NphaRFCS2), YP_326110; Halobacterium sp. strain NRC-1 RFCS2 (HspRFCS2), YP_280882; and H. walsbyi DSM RFCS2 (HwalRFCS2), YP_659090. Conserved and similar amino acid residues are black and gray, respectively. (B) Deletion of the insertion sequence in RFCS2Ma does not abolish the stimulation of DNA synthesis by the RFC Ma complex. Lane 1, template alone; lane 2, template/PolBI Ma; lane 3, template/PolBI Ma/PCNA Ma; lane 4, template/PolBI Ma/PCNA Ma/RFC Ma-RFC Ma RFCS2Δ complex; lane 5, lane 4 without PCNA Ma; lane 6, template/PolBI Ma/PCNA Ma/RFC Ma-RFC Ma wild type or positive control. The substrate for DNA synthesis was 0.5 μg of singly primed M13mp18 ssDNA in DNA polymerase reaction buffer. The reaction mixtures were incubated at 37°C for 5 min, the products were resolved by 1% alkali agarose gel electrophoresis, and signals were detected by autoradiography.
PolBlM0. Whereas the mutation in RFC box II (K18A) did not appear to impact the clamp loader’s capacity to stimulate PCNA-dependent DNA synthesis by PolBlM0 (Fig. 5B), the three remaining mutations completely abolished the capacity of the clamp loader to stimulate DNA synthesis by the cognate DNA polymerase in the presence of its clamp.

Hence, we demonstrate that, in addition to box III (Walker A motif) and box VII (SRC motif), the integrity of three other RFC boxes in the motor domain of the RFCM0 complex is critical to the clamp-loading reaction. These data agree with our recent biophysical analysis that captures the loading of a dye-labeled clamp to a dye-labeled primer/template junction of a DNA template (C. Liu et al., unpublished data). Similar assays have been used to observe clamp loading in other systems (36).

The RFCM0 complex represents the only biochemically characterized member of this new family of archaeal/eukaryotic RFC. This type of RFC is expected in most halochaeae, since the available genome sequences of representatives, such as Halobacterium sp. strain NRC-1, Haloarcula marismortui, Haloquadratum walsbyi, and Natronomonas pharaonis, have the three subunits in their genomes. In addition, several mesophilic methanogens, such as Methanospirillum hungatei and Methanothermobacter marisnigri, have the genes encoding orthologs of RFCM0 subunits. It is anticipated that the subunit organization, spatial distribution, and mutational data described for RFCM0 will be applicable to the clamp loader complex proteins in these organisms and other relatives yet to be described.

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REFERENCES


