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2 **Biochemical characterization and mutational analysis of the NurA protein from**
3 **the hyperthermophilic euryarchaeon *Thermococcus barophilus* Ch5**

4

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14

Abstract

Archaeal NurA protein plays a key role in producing 3'-single stranded DNA used for homologous recombination repair, together with HerA, Mre11, and Rad50. Herein, we describe biochemical characteristics and roles of key amino acid residues of the NurA protein from the hyperthermophilic euryarchaeon *Thermococcus barophilus* Ch5 (Tba-NurA). Tba-NurA possesses 5'-3' exonuclease activity for degrading DNA, displaying maximum efficiency at 45°C ~ 65°C and at pH 8.0 in the presence of Mn²⁺. The thermostable Tba-NurA also possesses endonuclease activity capable of nicking plasmid DNA and circular ssDNA. Mutational data demonstrate that residue D49 is essential for exonuclease activity and is involved in binding ssDNA since the D19A mutant lacked exonuclease activity and reduced ssDNA binding. No detectable dsDNA binding of the R96A and R129A mutants suggests that residues R96 and R129 are important for binding dsDNA. Since the D120A mutant had no degradation activity and reduced dsDNA binding, residue D120 is essential for degradation activities, and dsDNA binding. Additionally, residues Y392 and H400 are important for exonuclease activity since the mutations lead to exonuclease activity loss. To our knowledge, it is the first report on biochemical characterization and mutational analysis of the NurA protein from *Thermococcus*.

Keywords: NurA protein; Exonuclease; Endonuclease; Hyperthermophilic Archaea; DNA repair

1. Introduction

36

37 Double-strand breaks (DSBs) are extremely severe DNA lesions that are not only
38 caused by endogenous errors during replication and recombination, but also are
39 created by exogenous agents such as ionizing radiation and oxygen radicals [1]. If not
40 repaired, DSBs would lead to chromosome losses, deletions, and translocations [2-4],
41 thus posing severe damage to cells. Therefore, DSBs must be rapidly and accurately
42 repaired to maintain genome integrity and cell viability once they have been
43 produced.

44 Homologous recombination (HR) is one of the most efficient DSB repair
45 pathways, which utilizes a homologous template to repair DNA breaks in an
46 error-free way [5-6]. DNA ends are resected to form 3'-single-stranded DNA (ssDNA)
47 overhangs, which is an essential initiation step for loading recombinases during HR.
48 Various organisms utilize helicase and exonuclease to process DNA ends to initiate
49 HR. In bacteria, RecBCD/AddAB is the helicase-nuclease complex that unwinds and
50 degrades both DNA ends to produce 3'-ssDNA overhangs [7-10]. Besides, 5'-3'
51 exonuclease RecJ and helicase RecQ are also responsible for generating 3'-ssDNA
52 overhangs [11-12]. In eukaryotes, DSBs are initially recognized and processed by the
53 Mre11-Rad50-Nbs1 (in humans) or Mre11-Rad50-Xrs2 (in budding yeast) complex
54 [13-14]. Exo1 nuclease, Sgs1 helicase, and Dna2 nuclease are further recruited to
55 generate 3'-ssDNA overhangs [15]. In Archaea, eukaryotic-like Mre11-Rad50
56 complex initiates 5'-strand resection. However, Archaea lack eukaryotic Exo1, Dna2
57 and Sgs1 homologs, but possess 5'-3' exonuclease NurA and bidirectional DNA

58 helicase HerA that can produce 3'-ssDNA overhangs in addition to Mre11 and Rad50
59 [16-19]. Genomic analyses show that archaeal NurA, HerA, Mre11, and Rad50 share
60 an operon, thus suggesting that these four proteins are involved in DNA end resection
61 during archaeal HR [20-21].

62 NurA protein is widespread in Archaea and a small group of Bacteria. Since
63 *Sulfolobus acidocaldarius* NurA was firstly reported [16], five NurA homologs have
64 been characterized from Archaea to varying degrees: *Saccharolobus solfataricus* [18],
65 *Saccharolobus islandicus* [19], *Saccharolobus tokodaii* [22], *Pyrococcus furiosus* [20],
66 and *Thermococcus kodakaraensis* [23]. In Bacteria, the genomes of *Deinococcus* and
67 *Thermus* phyla also encode NurA protein, likely resulting from the gene transfer from
68 Archaea and Bacteria [24]. Notably, the activities of NurA proteins from Archaea and
69 Bacteria vary. While *S. acidocaldarius* NurA possesses endonuclease activity on
70 ssDNA and 5'-3' exonuclease activity on ssDNA and dsDNA [16], *P. furiosus* NurA
71 (Pfu-NurA) has weak 5'-3' exonuclease activity, but no endonuclease activity [20, 25].
72 *S. solfataricus* NurA, the high homology with *S. acidocaldarius* NurA, possesses 5'-3'
73 exonuclease activity on ssDNA and dsDNA and its exonuclease activity can be
74 modulated by HerA [26]. Additionally, genetic data show that *S. islandicus* NurA is
75 essential for cell viability [19]; however, *Thermus thermophilus* NurA is not essential
76 for cell viability [27]. Furthermore, the *nurA* gene knock-out *T. kodakaraensis* cells
77 are difficult to grow when exposed to DNA damaging agents [23]. Thus, distinct roles
78 of the *nurA* genes in archaeal and bacterial cells vary with source.

79 Currently, the biochemically characterized archaeal NurA proteins have

80 originated from Crenarchaea, especially from *Sulfolobus* species. Although cellular
81 function of *T. kodakaraensis* NurA protein was reported, no biochemical function of
82 NurA protein has been reported from *Thermococcus*. *Thermococcus barophilus* Ch5
83 is a hyperthermophilic euryarchaeon isolated from deep-sea, capable of growing
84 optimally at 88°C and 40 MPa pressure [28]. The genomic DNA of *T. barophilus* Ch5
85 encodes a putative NurA protein [29]. In this work, we revealed for the first time the
86 biochemical characteristics of degrading DNA by Tba-NurA. The roles of the
87 conserved residues in DNA degradation by Tba-NurA were also dissected by
88 mutational analyses.

89 **2. Materials and methods**

90 **2.1 DNA substrate**

91 The Hex-labeled oligonucleotide was synthesized at Sangon Biotech Company,
92 China. The sequence of the synthesized oligonucleotide is 5'-Hex- CGA ACT GCC
93 TGG AAT CCT GAC GAC ATG TAG CGA ACG ATC ACC TCA -3' and the
94 sequence of its complementary strand is 5'-TGA GGT GAT CGT TCG CTA CAT
95 GTC GTC AGG ATT CCA GGC AGT TCG -3'. The Hex-labeled DNA duplex was
96 prepared by annealing the synthesized oligonucleotide with its complementary strand
97 as described previously [30].

98 **2.2 Cloning, expression, and purification of Tba-NurA**

99 Using High-fidelity PCR Mix (Vazyme, Nanjing, China), the Tba-NurA gene
100 was amplified from the genomic DNA of *T. barophilus* Ch5 in the presence of the
101 forward and reverse primers containing the restriction sites of *Bam*HI and *Xho*I (Table

102 1). The PCR cycling program was followed as described in manual instruction of this
103 Kit. The PCR product and pET-28at-plus vector were cleaved by *Bam*HI and *Xho*I
104 and ligated by T4 DNA ligase to produce the recombinant plasmid harboring
105 Tba-NurA gene. The recombinant plasmid was transformed into the competent *E. coli*
106 BL21 (DE3) pLysS cells to express Tba-NurA protein after verification by
107 sequencing.

108 The expression strain was cultured in LB medium containing 34 µg/ml
109 chloramphenicol and 100 µg/ml kanamycin at 37°C. 0.1 mM isopropyl
110 thiogalactoside (IPTG) was added to the culture when the OD₆₀₀ value reached around
111 0.6. The culture continued to be shaken at room temperature for 12 hrs for protein
112 expression.

113 The cells were collected by centrifuge (6000g, 20 min), and the pellets were
114 resuspended with buffer A containing 20 mM Tris-HCl pH 8.0, 1 mM dithiothreitol
115 (DTT), 500 mM NaCl, 50 mM imidazole, and 10% glycerol. The cells were disrupted
116 by sonication. After centrifuge, the resulting supernatant was heated at 70°C for 20
117 min to denature the *E. coli* proteins. After centrifuge, the supernatant was filtered and
118 loaded onto a HisTrap FF column (GE Healthcare, Uppsala, Sweden) in NCG™
119 Chromatography System (Bio-Rad, CA, USA). The Tba-NurA protein was eluted
120 with a gradient of imidazole (50 mM ~ 500 mM) in buffer B containing 20 mM
121 Tris-HCl pH 8.0, 1 mM DTT, 500 mM NaCl, 500 mM imidazole, and 10% glycerol.
122 The fractions containing Tba-NurA protein were combined and dialyzed in a storage
123 buffer containing 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM DTT, and 50%

124 glycerol after the protein purity was verified by electrophoresis in 10% SDS-PAGE.
125 The purified Tba-NurA protein was kept at -80°C after dialysis. Based on its molar
126 extinction coefficient ($30,160\text{ M}^{-1}\text{ cm}^{-1}$), the Tba-NurA protein concentration was
127 calculated by measuring its absorbance at 280 nM.

128 **2.3 Modeling structure of Tba-NurA-ssDNA complex**

129 AlphaFold2 was used to model the Tba-NurA protein structure on the local
130 machine using its primary sequence [31]. The ssDNA was added to the model
131 structure by using the software Coot [32], based on the reported crystal structure of
132 *Thermotoga maritima* EndoV in complex with DNA (PDB ID code 2W36). And then
133 the conformations of the side chains of the residues in the modeled Tba-NurA-ssDNA
134 complex structure were slightly adjusted manually by using the software Coot [32].
135 The dimeric form of Tba-NurA was built through superposing onto the dimeric
136 structure of Pfu-NurA (PDB code: 3TAZ) [25]. Further any modification on the
137 original AlphaFold2-prediction structure was checked, regularized and validated. The
138 quality of the final structure was evaluated in Fig. S1 and Table S1 using MolProbity
139 [33]. The structural figures of the Tba-NurA-ssDNA complex model were finally
140 prepared with the Pymol software (<http://www.pymol.org>).

141 **2.4 Construction, overexpression, and purification of the Tba-NurA mutants**

142 Based on the wild-type (WT) plasmid containing Tba-NurA gene, the D49A,
143 R96A, D120A, R129A, Y392A and H400A mutants were constructed by employing
144 Site-directed Mutagenesis Kit (Transgen, China). The sequences of the mutagenic
145 primers for constructing these mutants are summarized in Table 1. The desired

146 mutations were confirmed by sequencing and then the Tba-NurA mutant proteins
147 were expressed and purified following the methods as described for the WT protein.

148 **2.5 Exonuclease activity assays**

149 In a standard exonuclease reaction (10 μ L), 100 nM 5'-Hex-labeled DNA or
150 3'-Hex-labeled DNA was incubated with 400 nM Tba-NurA in the buffer containing
151 20 mM Tris-HCl pH 8.0, 1 mM DTT, 8% glycerol, and 1 mM Mn^{2+} at 65°C for 30
152 min, unless stated. 98% formamide and 20 mM EDTA were added to the mixture to
153 stop the reaction. After heated at 95°C for 5 min, samples were separated by
154 electrophoresis in a denaturing 12% polyacrylamide gel with 8 M urea. The bands in
155 the gels were scanned and visualized with a molecular image analyzer (PharosFx
156 System, BioRad), and quantitated with ImageQuant software. The exonuclease
157 activity assays were replicated three times. Note that the reaction conditions distinct
158 from the standard reaction condition were shown in each figure legend.

159 **2.6 Endonuclease activity assays**

160 In an endonuclease reaction (10 μ L), 100 ng plasmid DNA or M13mp18 circular
161 ssDNA was incubated with Tba-NurA with varying concentrations in the buffer
162 containing 20 mM Tris-HCl pH 8.0, 1 mM DTT, 8% glycerol, and 1 mM Mn^{2+} at
163 65°C for 30 min. The endonuclease reaction was terminated with the addition of 98%
164 formamide and 20 mM EDTA. Samples were separated by electrophoresis in a
165 denaturing 0.7% agarose gel. The bands in the gels were scanned and visualized with
166 a molecular image analyzer.

167 **2.7 DNA binding assays**

168 100 nM 5'-Hex-labeled ssDNA or dsDNA was incubated with the WT/mutant
169 Tba-NurA with varying concentrations in the buffer containing 20 mM Tris-HCl pH
170 8.0, 1 mM DTT, and 8% glycerol at room temperature for 10 min. Samples were
171 analyzed by electrophoresis in a native 4% polyacrylamide gel with 0.1 x TBE. The
172 gels were treated as described above. The DNA binding assays were replicated three
173 times.

174 3. Results

175 3.1 Tba-NurA is a putative 5'-3' exonuclease

176 Sequence alignment of Tba-NurA and other characterized NurA proteins shows
177 that Tba-NurA possesses three conserved motifs: Motif I (AxDG), Motif II (xxxDGx),
178 and Motif III (GYxxPLxxxH), where x represents any amino acid residue (Fig. 1).
179 Tba-NurA and other NurA proteins from Euryarchaea harbor a M domain. However,
180 this M domain is absent in the NurA proteins from Crenarchaea and Bacteria.
181 Tba-NurA displays more amino acid similarity to *T. kodakaraensis* NurA (58%) than
182 Pfu-NurA (38%). These observations suggest that Tba-NurA should degrade DNA in
183 the 5'-3' direction since it possesses the conserved motifs that associates with 5'-3'
184 exonuclease activity. On the other hand, 17% ~ 20% amino acid similarity is observed
185 between Tba-NurA and the NurA homologues from *D. radiodurans*, *S. islandicus*, *S.*
186 *acidocaldarius*, *S. solfataricus* and *S. tokodaii*, which suggests that Tba-NurA might
187 harbor distinct biochemical characteristics from other NurA proteins.

188 To probe biochemical characteristics of Tba-NurA, we cloned its gene, expressed
189 and purified this protein (Fig. 2). The recombinant Tba-NurA gene was successfully

190 induced for expression as a ~ 51 kDa protein containing a 6 x His-tag at its
191 N-terminus (Fig. 2). Through the following purification steps including sonication,
192 heat treatment (70°C for 20 min), and Ni column affinity chromatography, the
193 recombinant Tba-NurA protein was finally purified (Fig. 2).

194 **3.2 Tba-NurA possesses 5'–3' exonuclease activity**

195 After successfully purifying the Tba-NurA protein, we first determined whether
196 this protein can degrade DNA by performing exonuclease reactions using
197 3'-Hex-labeled or 5'-Hex-labeled ssDNA and dsDNA as substrates. The
198 3'-Hex-labeled ssDNA substrate was gradually degraded by Tba-NurA as the enzyme
199 concentrations increased at 65°C (Fig. 3A). Specifically, above 90% of the substrate
200 was degraded by 400 nM enzyme. Similar degradation results were observed for
201 3'-Hex-labeled dsDNA (Fig. 3B). However, the enzyme displayed the lower
202 efficiency for degrading 3'-Hex-labeled dsDNA than that for degrading
203 3'-Hex-labeled ssDNA at 100 nM or 200 nM Tba-NurA. Moreover, we also found
204 that the enzyme exhibited similar degradation efficiencies for 5'-Hex-labeled ssDNA
205 and dsDNA as observed for 3'-Hex-labeled ssDNA and dsDNA (Figs. 3C and 3D),
206 respectively. While only one band appeared in the 5'-Hex-labeled ssDNA cleavage
207 reactions (Fig. 3C), several bands were observed for degrading 3'-Hex-labeled ssDNA
208 (Fig. 3A), thus confirming that this protein possesses 5'–3' exonuclease activity.
209 Therefore, Tba-NurA is a 5'–3' exonuclease, capable of not only efficiently degrading
210 ssDNA, but also degrading dsDNA with the relatively reduced efficiency.

211 **3.3 Tba-NurA possesses endonuclease activity**

212 *S. solfataricus* NurA possesses endonuclease activity [18], which prompted us to
213 investigate whether Tba-NurA has endonuclease activity. We employed plasmid
214 cccDNA and circular ssDNA as substrates to perform endonuclease activity reactions
215 of Tba-NurA, respectively. Compared with the control reaction (CK1) without the
216 heating treatment (Fig. 4A), slight ocDNA (open circular, ocDNA) was produced
217 from the cccDNA (covalent closed circular DNA) substrate by the incubation at 65°C
218 for 30 min (CK2), demonstrating that most of cccDNA is stable when kept at 65°C for
219 30 min. On the other hand, more ocDNA and linear DNA were created by Tba-NurA,
220 demonstrating that the enzyme possesses endonuclease activity that can nick
221 cccDNA.

222 To further confirm endonuclease activity of Tba-NurA, we investigated whether
223 this enzyme can nick circular ssDNA. As shown in Fig.4B, the circular ssDNA
224 substrate is stable after incubated at 65°C for 30 min since no band intensity change
225 was observed in the CK2 with heating treatment and CK1 without heating treatment.
226 Interestingly, the circular ssDNA substrate was degraded to be short DNA fragments
227 by Tba-NurA at the tested enzyme concentrations. The circular ssDNA was first
228 cleaved by Tba-NurA's endonuclease activity to form linear ssDNA and then this
229 ssDNA product was further degraded by its exonuclease activity to generate smear
230 DNA fragments. Thus, these observations demonstrate that Tba-NurA harbors
231 endonuclease activity that nicks plasmid DNA and circular ssDNA in addition to 5'-3'
232 exonuclease activity.

233 **3.4 Biochemical characteristics of Tba-NurA**

234 Since Tba-NurA can efficiently degrade ssDNA in the direction of 5'–3', we used
235 the 5'-labeled ssDNA as substrate to determine its biochemical characteristics. Firstly,
236 we examined the degradation efficiency of the enzyme at various temperatures, and
237 found that Tba-NurA can degrade this ssDNA substrate at the tested temperatures
238 ranging from 35°C to 95°C with different cleavage efficiencies (Fig. 5A). At 45°C ~
239 65°C, the enzyme displayed maximum degradation efficiency, demonstrating that
240 45°C ~ 65°C is its optimal temperature for cleaving ssDNA.

241 The thermo-tolerance of the Tba-NurA protein was assayed by determining the
242 heated enzyme activity. Tba-NurA retained above 90% degradation activity after
243 heated at 75°C and 80°C for 20 min (Fig. 5B), which is similar to that of the control
244 reaction performed by the non-heated enzyme. As the heating temperature increased
245 from 85°C to 95°C, the enzyme displayed reduced degradation efficiency. Specifically,
246 the enzyme still possessed 34% degradation percentage even after a 20 min treatment
247 at 95°C, suggesting that the enzyme is quite thermostable.

248 We also examined the effect of pH on the Tba-NurA activity. Various
249 degradation efficiencies were observed at pH 7.0 to 9.0 (Fig. 5C). Maximum
250 degradation efficiency of the enzyme was observed at pH 8.0. By comparison, no
251 ssDNA substrate was degraded by the enzyme at pH 10.0 and 11.0. Additionally, this
252 substrate was slightly degraded by Tba-NurA at pH 7.0. Thus, these observations
253 show that the optimal pH of Tba-NurA activity is 8.0.

254 Previous studies show that Mn^{2+} is needed for the NurA activity [34], which
255 prompted us to investigate whether a divalent ion is required for Tba-NurA to degrade

256 ssDNA. In the first control reaction without the addition of any divalent metal ion, no
257 ssDNA substrate was degraded by the enzyme (Fig. 5D). In the second control
258 reaction with the addition of 10 mM EDTA, no DNA substrate was still degraded,
259 suggesting that a divalent metal ion is needed for Tba-NurA to degrade ssDNA. As
260 expected, Tba-NurA displayed maximum degradation efficiency with 1 mM Mn^{2+} . By
261 comparison, the enzyme had no activity with Ca^{2+} , Cu^{2+} , Zn^{2+} or Ni^{2+} . Additionally,
262 the enzyme exhibited weak activity with Mg^{2+} . Therefore, these observations show
263 that Tba-NurA is Mn^{2+} -dependent.

264 **3.5 Homology structure of the Tba-NurA-ssDNA complex**

265 The model structure of Tba-NurA-ssDNA complex was constructed by using
266 AlphaFold2 (Fig. 6A), possessing three conserved motifs present in other reported
267 NurA proteins. We compared our structural model with the two reported crystal
268 structures of the archaeal NurA proteins from *P. furiousus* (PDB code: 3TAZ) and *S.*
269 *solfatarius* (PDB code: 2YGK) [25, 35]. The M domains in the middle of the
270 structures show significant conformational difference between Pfu-NurA and
271 Tba-NurA (Fig. 5B). In the above two structures, the M-domain forms a peak and the
272 other part forms a base of a pyramid [25]. Our model shows a symmetric peak
273 whereas Pfu-NurA displays a twisted asymmetric peak (Fig. 6B). In contrast, NurA
274 from *S. solfataricus* lacks the M domain (Fig. 6C). Additionally the structure shows
275 the H5 helix is much shorter than the other two structures.

276 The motif I in Tba-NurA contains “AxDG”, among which residue D49 is highly
277 conserved among the NurA proteins except *T. thermophilus* NurA protein.

278 Additionally, residue D120 of the conserved “DG” in motif II of the enzyme is
279 invariable among all the reported NurA proteins. Furthermore, residues Y392 and
280 H400 of the GYxxPLxxAH in motif III of Tba-NurA are highly conserved. Although
281 residues R96 and R129 of Tba-NurA are not conserved, they are close to ssDNA
282 substrate, suggesting that they might play a role in cleaving DNA.

283 To clarify the roles of these six conserved residues of Tba-NurA in degrading
284 DNA and binding DNA, we needed to characterize the Tba-NurA D49A, R96A,
285 D120A, R129A, Y392A and H400A mutants biochemically. By using the WT
286 plasmid harboring the Tba-NurA gene as the template, the mutant plasmids were
287 successfully constructed via site-directed mutagenesis. The Tba-NurA mutant proteins
288 were also successfully expressed and purified as described for the WT protein (Fig.
289 S2).

290 **3.6 DNA degradation of the Tba-NurA mutants**

291 Under the optimized DNA degradation reaction condition as described above, we
292 determined the degradation efficiencies of the Tba-NurA D49A, R96A, D120A,
293 R129A, Y392A and H400A mutants using various DNA substrates. Compared with
294 the WT protein, the D49A, D120A, Y392A, and H400A mutants almost completely
295 abolished the degradation activity when using 5'-Hex-labeled ssDNA as substrate (Fig.
296 7A), thereby demonstrating that residues D49, D120, Y392, and H400 are essential
297 for degrading ssDNA. Furthermore, the R96A and R129A mutants displayed 39% and
298 32% degradation, thus showing that residues R96 and R129 have partial contribution
299 to degrading ssDNA. Similar degradation results of all Tba-NurA mutants were

300 observed for 3'-Hex-labeled ssDNA (Fig. S3). Moreover, we also found that all
301 Tba-NurA mutants had no detectable exonuclease activity for 3'-Hex-labeled and
302 5'-Hex-labeled dsDNA (Figs. 7B and S4), demonstrating that these tested six residues
303 are important for degrading dsDNA.

304 Using plasmid cccDNA and circular ssDNA as substrates, we also investigated
305 endonuclease activities of Tba-NurA mutants. We found that the D49A, Y392A and
306 H400A mutants had the WT protein activity (Fig. 7C), indicating that residues D49,
307 Y392 and H400 is not involve in nicking plasmid cccDNA. However, compared with
308 the WT protein, the R96A, D120A and R129A mutants possessed the reduced nicking
309 activity. On the other hand, we found that all Tba-NurA mutants displayed weak
310 endonuclease activity for circular ssDNA since slight ssDNA substrate was degraded
311 by these mutants (Fig. 7D).

312 **3.7 DNA binding of the Tba-NurA mutants**

313 Since Tba-NurA can degrade ssDNA with high efficiency, we tested whether this
314 exonuclease can bind DNA. We found that the ssDNA substrate was gradually bound
315 by Tba-NurA as the enzyme concentrations increased (Fig. 8A). At 2000 nM, the
316 ssDNA substrate was almost completely bound by Tba-NurA. Furthermore,
317 Tba-NurA can bind dsDNA substrate with almost same efficiency at 2000 nM
318 enzyme as observed for binding ssDNA substrate (Fig. 8A). However, compared with
319 the binding ssDNA, the enzyme displayed the reduced efficiency for binding dsDNA
320 when 500 nM and 1000 nM Tba-NurA concentrations were used. Thus, our
321 observations show that Tba-NurA can not only bind ssDNA , but also bind dsDNA

322 with reduced efficiency.

323 To probe the roles of these six conserved residues in binding ssDNA, we also
324 investigated the binding efficiency of the Tba-NurA mutants. As shown in Fig. 8B,
325 the R96A, D120A, R129A, Y392A and H400A mutants displayed the WT protein
326 binding efficiency, thus suggesting that residues R96, D120, R129, Y392 and H400
327 are not essential for binding ssDNA. However, the D49A mutant retained the reduced
328 binding efficiency relative to the WT protein, demonstrating that residue D49 is
329 involved in binding ssDNA.

330 On the other hand, we found that the D49A mutant retained the WT protein
331 efficiency for binding dsDNA (Fig. 8C), suggesting that residue D49 is not essential
332 for binding dsDNA. The Y392A and H44A mutants displayed increased efficiency for
333 binding dsDNA relative to the WT protein, which indicates that residues Y392 and
334 H44 are not involved in binding dsDNA. By contrast, the R96A, D120A and R129
335 mutants abolished the ability to bind dsDNA, suggesting that residues R96, D120, and
336 R129 are responsible for binding dsDNA.

337 **4. Discussion**

338 **4.1 Tba-NurA possesses 5'–3' exonuclease and endonuclease activities**

339 Archaeal NurA proteins are widespread in Crenarchaea and Euryarchaea, but
340 most studies focus on crenarchaeal NurA proteins, especially from *Saccharolobus*. In
341 this work, we report that the NurA protein from *T. barophilus* Ch5 can degrade
342 ssDNA and dsDNA in the 5'–3' direction, and also nick plasmid DNA and circular
343 ssDNA as well, demonstrating that the enzyme possesses 5'–3' exonuclease and

344 endonuclease activities.

345 Although the enzyme is more homologous with Pfu-NurA than the NurA
346 proteins from *S. acidocaldarius* and *S. tokodaii*, Tba-NurA resembles the NurA
347 proteins from *S. acidocaldarius* and *S. tokodaii* in terms of activities [16, 22], but
348 contrasts with the Pfu-NurA [20, 25]. Comparison of biochemical characteristics of
349 these two NurA proteins is summarized in Table 2. *S. tokodaii* NurA (Sto-NurA) is a
350 well characterized protein [22, 34-35], possessing endonuclease and 5'-3' exonuclease
351 activities as observed for Tba-NurA. Tba-NurA displays the same optimal
352 temperature (80°C) as observed for Sto-NurA [34]. However, Tba-NurA retains 81%
353 activity while Sto-NurA exhibits 25% activity after heated 90°C for 20 min [35],
354 demonstrating that the former is more thermostable than the latter. Additionally, both
355 Tba-NurA and Sto-NurA are Mn²⁺-dependent and display the same optimal pH.

356 **4.2 The roles of conserved residues of Tba-NurA**

357 In this work, we systemically revealed exonuclease and endonuclease activities,
358 and DNA binding of the Tba-NurA D49A, R96A, D120A, R129A, Y392A and
359 H400A mutants to clarify the roles these six residues (Table 3). Residue D49 in motif
360 I of Tba-NurA is highly conserved in the characterized NurA proteins except *T.*
361 *thermophilus* NurA (Fig. 1). Our mutational data confirm that residue D49 of
362 Tba-NurA is essential for exonuclease activity. The crystal structure of the
363 Pfu-NurA-dAMP-Mn²⁺ complex shows that the first Mn²⁺ site is coordinated by
364 residue D51 (residue D49 in Tba-NurA) [25], suggesting that the activity loss of the
365 D49A mutant might be due to the disruption of coordination with Mn²⁺. Similar

366 results were observed for the Sto-NurA D49A, *D. radiodurans* NurA D53A, *S.*
367 *olfataricus* NurA D58A mutants [22, 24, 26]. Additionally, we found that residue
368 D49 is involved in binding ssDNA and nicking circular ssDNA as well). Overall, the
369 highly conserved Asp in motif I of NurA proteins plays a role in degrading DNA, but
370 provide evidence that it is responsible for ssDNA binding.

371 Like residue D51, residue D126 of Pfu-NurA coordinates with the first Mn^{2+} in
372 the Pfu-NurA-dAMP- Mn^{2+} complex [25], which indicates that residue D126 might be
373 also another catalytic residue. We found that residue D120 of Tba-NurA (residue D51
374 in Pfu-NurA) is essential for degrading DNA since the loss activity of the D120A
375 mutant might be caused by this disruption of coordination with Mn^{2+} . Similar roles
376 are observed for Sto-NurA D131 [22], *D. radiodurans* NurA D184 [24], and *S.*
377 *olfataricus* NurA D133 [26]. However, residue D120 is important for binding
378 dsDNA rather than for binding ssDNA (Fig. 8).

379 The structure of the Pfu-NurA-dAMP- Mn^{2+} complex demonstrates that Y403 is
380 one of the key residues that associate with DNA substrate [25]. Mutational analysis
381 shows that residue Y403 is responsible for DNA binding [25]. By comparison, our
382 binding data show that residue Y392 of Tba-NurA (residue Y403 of Pfu-NurA) is not
383 essential for DNA binding (Fig. 7). Furthermore, our mutational data show that the
384 Y392A mutant completely abolished the degradation activity, which could be because
385 the Y392A mutation might disrupt the contact between residue Y392 and residue
386 D120. This hypothesis can be supported by this observation that residue Y298 of *S.*
387 *olfataricus* NurA (residue Y392 in Tba-NurA) contacts residue D133 (residue D120

388 in Tba-NurA) by its side chain towards the active site [26]. Interestingly, destabilize a
389 reaction intermediate preventing the reaction from proceeding. However, the
390 Sto-NurA Y291A still retains partial activity relative to the WT protein [22]. Thus,
391 the function of the conserved Tyr in the motif III of NurA varies with its source.

392 Residue H411 of Pfu-NurA is one of the key residues that coordinate with a
393 second Mn^{2+} [25], suggesting that residue H411 might be another catalytic residue. In
394 this work, we found that residue H400 of Tba-NurA (residue H411 of Pfu-NurA) is
395 necessary to degrade DNA (Fig. 8). Similar role are observed for the corresponding
396 residue H299 of Sto-NurA [22]. However, residue H400 of Tba-NurA is not involved
397 in binding DNA (Fig. 8).

398 Residue R96 of Tba-NurA is conserved in NurA proteins from Euryarchaea and
399 *D. radiodurans* NurA, but not in NurA proteins from Crenarchaea and *T.*
400 *thermophilus* NurA. The structure of the Pfu-NurA-dAMP- Mn^{2+} complex displays
401 that residue R98 (residue of R96 of Tba-NurA) might stabilize the substrate or
402 reaction intermediate [25]. In this work, we revealed that residue R96 is involved in
403 degrading DNA. This mutation destabilized a reaction intermediate preventing the
404 reaction from proceeding. Interestingly, residue R96 is important for dsDNA binding
405 rather than for ssDNA binding (Fig. 7). To our knowledge, it is the first report on the
406 role of the conserved arginine in euryarchaeal NurA proteins.

407 Like residue R98, residue R135 of Pfu-NurA stabilizes the substrate or reaction
408 intermediate [25]. Likewise, residue R135 of Pfu-NurA is conserved in euryarchaeal
409 NurA proteins, *S. acidocaldarius* NurA and *D. radiodurans* NurA. In this work, we

410 found that residue R129 of Tba-NurA (residue R135 of Pfu-NurA) is involved in
411 degrading DNA. Like residue R96, residue 129 is important for dsDNA binding rather
412 than for ssDNA binding (Fig. 7). This is also the first report on the role of the
413 conserved arginine in eurychaean NurA proteins.

414 **4.3 Possible physiological role of Tba-NurA**

415 Like other Archaea, the genome of the *T. barophilus* Ch5 encodes HerA (NCBI:
416 ALM75038), Mre11 (NCBI: ALM75037) and Rad50 (NCBI: ALM75036) proteins in
417 addition to NurA [29]. Although the genomic organization of these four genes in *T.*
418 *barophilus* is *nurA*, *rad50*, *mre11* and *herA* (Fig. S5), which is same as that of *T.*
419 *kodakarensis* but is distinct from that of other Archaea. These four proteins share an
420 operon, which suggests that a combination of these four proteins is responsible for
421 generating ssDNA 3'-overhang used for HR although the HerA, Mre11 and Rad50
422 proteins have not been characterized. In this work, our biochemical data have
423 confirmed the 5'-3' ssDNA and dsDNA degradation activity of Tba-NurA protein,
424 thereby indicating that this protein might play a role in producing ssDNA for HR.

425 The structures of the archaeal NurA proteins from *P. furiousus* [25] and *S.*
426 *solfataricus* were solved [26, 36], possessing an argonaute-, endoV nuclease- and
427 RNase-like domain, which are similar to the structure of the bacterial NurA protein
428 from *D. radiodurans* [37]. The *P. furiousus* NurA structure is folded in a
429 pyramid-shaped dimer harboring a central channel. The central channel of the NurA
430 dimer is too narrow to accommodate for dsDNA but well for ssDNA or one strand of
431 an unwound duplex. Our biochemical data demonstrate that Tba-NurA possesses

432 reduced dsDNA degradation activity relative to ssDNA degradation activity, which
433 provides well support for structural characteristic. Additionally, reduced dsDNA
434 degradation activity and strong ssDNA degradation activity of Tba-NurA suggests
435 that Tba-HerA might be needed to associate with this enzyme to complete DSB repair
436 in vivo. We are characterizing Tba-HerA to probe DSB repair by HR in this archaeon.
437 It would be interesting to investigate *in vivo* interaction between NurA and HerA in
438 this organism to determine whether NurA is essential for viability in *T. barophilus*.

439 **5. Conclusion**

440 In summary, we report biochemical characteristics of Tba-NurA and the roles of
441 its six conserved residues in DNA degradation. Tba-NurA can degrade ssDNA and
442 dsDNA in the direction of 5'–3' at high temperature, displaying higher degradation
443 efficiency for ssDNA than that for dsDNA. The enzyme also possesses endonuclease
444 activity capable of nicking plasmid cccDNA and circular ssDNA. Furthermore, the
445 enzyme displays several biochemical characteristics distinct from the reported NurA
446 proteins. Mutational data demonstrate that residue D49 is essential for exonuclease
447 activity and ssDNA binding, residues R96 and R129 are important for binding
448 dsDNA, residue D120 is essential for exonuclease and endonuclease activities, and
449 dsDNA binding, and residues Y392 and H400 are important for exonuclease activity.

450 **Author Contributions**

451 LZ and PO wrote the main manuscript text. LZ prepared figure 1. LZ, GM, KD,
452 LM and TL prepared figures 2-5 and 7-8. PC prepared figure 6. LZ, GM and TL
453 prepared all supplemental figures. All authors reviewed the manuscript.

454

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458

Declaration of Competing Interest

459 All authors declare that there is no conflict of interests regarding the publication
460 of this paper.

461

Data availability

462 All data generated or analyzed during this study are included in this published
463 article and its supplementary information files.

464

Ethical approval

465 This article does not contain any studies with human participants or animals
466 performed by any of the authors.

467

Figure legends

468 **Fig. 1** Multiple sequence alignment of the characterized NurA proteins. Tba:
469 *Thermococcus barophilus* (ALM75035); Tko: *Thermococcus kodakarensis*
470 (WP_011251160); Sis: *Saccharolobus islandicus* REY15A (ADX84156); Sac:
471 *Saccharolobus acidocaldarius* (CAD26846); Sso: *Saccharolobus solfataricus*
472 (WP_009991544); Sto: *Saccharolobus tokodaii* (BAB67213); Pfu: *Pyrococcus*
473 *furiosus* (WP_011012308); Tth: *Thermus thermophilus* (QMV31481); Dra:
474 *Deinococcus radiodurans* (WP_010887482). Similar or conserved amino acid
475 residues are boxed and colored. The replaced residues are shown with “*”.

476 **Fig. 2** Expression and purification of Tba-NurA protein. The gene encoding
477 Tba-NurA protein was induced for expression with the addition of IPTG and the
478 expressed gene product was purified via sonication, heat treatment (70°C for 20 min)
479 and Ni column affinity chromatograph.

480 **Fig. 3** Exonuclease activity assays of Tba-NurA. DNA degradation reactions were
481 performed with 100, 200 and 400 nM Tba-NurA at 65°C for 30 min using various
482 DNA substrates. A. Degradation of the 3'-labeled ssDNA. B. Degradation of the
483 3'-labeled dsDNA. C. Degradation of the 5'-labeled ssDNA. D. Degradation of the
484 5'-labeled dsDNA. CK: the reaction without Tba-NurA.

485 **Fig. 4** Endonuclease activity assays of Tba-NurA. DNA degradation reactions were
486 performed with 250, 500 and 1000 nM Tba-NurA at 65°C for 30 min using plasmid
487 cccDNA and circular ssDNA as substrates. A. Degradation of plasmid cccDNA. B.
488 Degradation of circular ssDNA. CK1: the reaction without Tba-NurA and without
489 heating treatment. CK2: the reaction without Tba-NurA but with heating treatment. M:
490 DNA marker.

491 **Fig. 5** Biochemical characterization assays of Tba-NurA. The ssDNA substrate was
492 used as substrate to determine biochemical characterization of Tba-NurA under
493 different condition. Samples were separated by electrophoresis in a denaturing 12%
494 gel. A. The determination of optimal temperature. DNA degradation reactions were
495 performed at 35°C, 45°C, 55°C, 65°C, 75°C, 85°C and 95°C for 30 min, respectively.
496 B. The thermo-tolerance assays. Tba-NurA was heated at 75°C, 80°C, 85°C, 90°C and
497 95°C for 20 min and then used to degrade ssDNA substrate, respectively. C. The

498 determination of optimal pH. DNA degradation reactions were performed at pH 7.0,
499 pH 7.5, pH 8.0, pH 8.5, pH 9.0, pH 10.0, and pH 11.0. The desired pHs were made
500 with five different buffers (all at 20 mM concentrations): Tris-HCl (pH 7.0, pH 7.5,
501 pH 8.0, and pH 8.5), and Gly-NaOH (pH 9.0, pH 10.0 and pH 11.0). D. The divalent
502 ion effect assays. DNA degradation reactions were performed with various divalent
503 ions (1 mM). CK in the panels A and C, and CK1 in the panels B and D: the reaction
504 without Tba-NurA. CK2 in the panel B: the reaction with unheated Tba-NurA; CK2
505 in the panel D: the reaction without a divalent ion.

506 **Fig. 6** Structure comparison of homology model of the Tba-NurA-ssDNA complex
507 with the other NurA structures. A. Homology model of the Tba-NurA-ssDNA
508 complex. The motifs I, II and III, and M domain in Tba-NurA are colored with
509 magentas, yellow, green, and cyan, respectively. The whole monomer section of
510 Tba-NurA dimer is shown with pink. The mutated residues are shown with the sticks
511 with different colors. B. Structure comparison of the model Tba-NurA-ssDNA
512 complex with Pfu-NurA. Our model (in blue) was superposed onto the crystal
513 structure of Pfu-NurA (PDB code: 3TAZ) (in yellow). The ssDNA (in green) was
514 sandwiched between two chains. C. Structure comparison of the model
515 Tba-NurA-ssDNA complex with *S. solfataricus* NurA. Our model (in blue) was
516 superposed onto the crystal structure of *S. solfataricus* NurA (PDB code: 2YGK) (in
517 red). The M domain is shown in a red dashed circle and in two black rectangular
518 frames in the panels B and C.

519 **Fig. 7** Exonuclease and endonuclease activity assays of the Tba-NurA mutants.
520 Various DNA substrates were used to investigate exonuclease and endonuclease
521 activities of the Tba-NurA mutants. A. 5'-Hex-labeled ssDNA degradation result of
522 the Tba-NurA mutants. B. 5'-Hex-labeled dsDNA degradation result of the Tba-NurA
523 mutants. C. Plasmid cccDNA degradation result of the Tba-NurA mutants. D.
524 Circular ssDNA degradation result of the Tba-NurA mutants. The WT protein was
525 used for comparison. CK: the reaction without the Tba-NurA mutants.

526 **Fig. 8** DNA binding assays of the WT/mutant Tba-NurA. Various DNA substrates
527 were used to determine DNA binding of the WT/mutant Tba-NurA at room
528 temperature. A. 5'-Hex-labeled ssDNA and dsDNA binding result of Tba-NurA. B.
529 5'-Hex-labeled ssDNA binding result of the WT/mutant Tba-NurA. C. 5'-Hex-labeled
530 dsDNA binding result of the WT/mutant Tba-NurA. CK: the reaction without
531 Tba-NurA.

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651

Table 1 Oligonucleotides used in this work

Name	Sequence (5'-3')
Tba-NurA F	CGC <u>GGATCC</u> ATGTATCGCCTAATTTCAAAG
Tba-NurA R	CCGCTCGAGTCACTCCAAAGGCGACCTTCC
D49A F	CAAAGTTTATGCTGTTGCTGGCAGTCAAGGT
D49A R	GCAACAGCATAAACTTTGCTCTCTTTCTTTT
R96A F	TCTCTGACCAGATAATCGCGTTGCAGATGGAA
R96A R	GCGATTATCTGGTCAGAGATTCCATGATTGTA
D120A F	GCACATGATTTTGATGGCCGGAACCCTTACA
D120A R	GCCATCAAAATCATGTGCTTTTCAGTCCCAA
R129A F	TTACAGGTTCTTTAACG GC ACCTCCAGTTTAT
R129A R	GCCGTTAAAGAACCTGTAAGGGTTCCGTCCAT
Y392A F	GGCATGAGAGCAGCGGG GCT ATAAGGCCTCTT
Y392A R	GCCCCGCTGCTCTCATGCCAGAGGAGCTTTCC
H400A F	GGCCTCTTCAGAGGGCG GCT GAAGGAGTAAAG
H400A R	GCCGCCCTCTGAAGAGGCCTTATATACCCGCT

652 The underlined bases represent restriction sites of *Bam*HI and *Xho*I.

653 The substitution bases are italic and bolded.

654

655

656

Table 2 Comparison of biochemical characteristics of Tba-NurA and Sto-NurA

NurA protein	Optimal Temp (°C)	Thermostability	pH	Divalent ion requirement	Reference
Tba-NurA	45 ~ 65	Remaining 81% activity after heating 90°C for 20 min	8.0	Mn ²⁺ -dependent	This work
Sto-NurA	65	Remaining 25% activity after heating 90°C for 20 min	8.0	Mn ²⁺ -dependent	[34]

Table 3 Comparison of exonuclease and endonuclease activity and DNA binding of the WT and mutant Tba-NurA

Tba-NurA	ssDNA exonuclease activity	dsDNA exonuclease activity	Nicking plasmid cccDNA activity	Nicking circular ssDNA activity	Binding ssDNA	Binding dsDNA
WT	++++ ^a	+++	+++	+++	+++	++
D49A	-	-	+++	+	+	++
R96A	++	-	+	+	+++	-
D120A	-	-	+	+	+++	-
R129A	++	-	+	+	+++	-
Y392A	-	-	++	+	+++	+++
H400A	-	-	++	+	+++	+++

++++: very strong activity; +++: strong activity; ++: moderate activity; +: weak activity; -: no detectable activity.