Biochemical and Functional Characterization of the NurA-HerA Complex from *Deinococcus radiodurans*

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ABSTRACT

In archaea, the NurA nuclease and HerA ATPase/helicase, together with the Mre11-Rad50 complex, function in 3' single-stranded DNA (ssDNA) end processing during homologous recombination (HR). However, bacterial homologs of NurA and HerA have not been characterized. From *Deinococcus radiodurans*, we identified the manganese-dependent 5'-to-3' ssDNA/double-stranded DNA (dsDNA) exonuclease/endonuclease NurA (DrNurA) and the ATPase HerA (DrHerA). These two proteins stimulated each other's activity through direct protein-protein interactions. The N-terminal HAS domain of DrHerA was the key domain for this interaction. Several critical residues of DrNurA and DrHerA were verified by site-directed mutational analysis. Temperature-dependent activity assays confirmed that the two proteins had mesophilic features, with optimum activity temperatures 10°C to 15°C higher than their optimum growth temperatures. Knocking out either *nurA* or *herA* affected cell proliferation by shortening the growth phase, especially for growth at a high temperature (37°C). In addition, both mutant strains displayed almost 10-fold-reduced intermolecular recombination efficiency, indicating that DrNurA and DrHerA might be involved in homologous recombination *in vivo*. However, single- and double-gene deletions did not show significantly decreased radioresistance. Our results confirmed that the biochemical activities of bacterial NurA and HerA proteins were conserved with archaea. Our phenotypical results suggested that these proteins might have different functions in bacteria.

IMPORTANCE

Deinococcus radiodurans NurA (DrNurA) was identified as a manganese-dependent 5'-to-3' ssDNA/dsDNA exonuclease/endonuclease, and *Deinococcus radiodurans* HerA (DrHerA) was identified as an ATPase. Physical interactions between DrNurA and DrHerA explained mutual stimulation of their activities. The N-terminal HAS domain on DrHerA was identified as the interaction domain. Several essential functional sites on DrNurA and DrHerA were characterized. Both DrHerA and DrNurA showed mesophilic biochemical features, with their optimum activity temperatures 10°C to 15°C higher than their optimum growth temperatures *in vitro*. Knockout of *nurA* or *herA* led to abnormal cell proliferation and reduced intermolecular recombination efficiency but no obvious effect on radioresistance.

DNA double-strand breaks (DSBs) cause chromosome losses or deletions, translocations, and genomic instability and profoundly influence the proliferation of normal cells. Homologous recombination (HR) generates error-free repair products and is one of the most important DSB repair pathways (1, 2). During HR initiation, DNA ends are processed into 3'-single-stranded DNA (ssDNA) overhangs that are necessary for loading recombinases (RecA/Rad51/RadA) (3, 4).

In eukaryotes, Mre11-Rad50-Nbs1 (human) or Mre11-Rad50-Xrs2 (*Saccharomyces cerevisiae*) in conjunction with Ctp1/CtIP (or Sae2) initially recognizes DSBs and removes small nucleotides to form early intermediates during HR (5–11). Exo1 nuclease, Sgs1 helicase, and Dna2 nuclease bind to these intermediates and generate 3' ssDNA overhangs (1, 7, 9–11). In archaea, the Mre11-Rad50 complex also senses and processes DSB ends through 3'-to-5' exonuclease and endonuclease activities to form short 3' overhangs (12, 13). No Exo1, Sgs1, or Dna2 homologs have been found in archaea, and NurA and HerA are believed to be replacements (13, 14). *nurA* and *herA* are usually located in operons. The *nurA* gene encodes a 5'-to-3' ssDNA/dsDNA exonuclease/endonuclease (15), and *herA* encodes an ATP-dependent bipolar helicase that unwinds dsDNA in both the 5'-to-3' and 3'-to-5' directions (16, 17). Usually, *mre11-rad50* colocalizes with this operon (18), and *nurA*, *herA*, *mre11*, and *rad50* are cotranscribed in re-

sponse to UV irradiation (19, 20). Functional interactions between NurA-HerA and Mre11-Rad50 indicate that these four proteins are involved in HR DNA end resection (13, 21).

In bacteria, RecBCD/AddAB enzyme family members have helicase and 3'-to-5' and 5'-to-3' exonuclease activities during 3' ssDNA end resection (22–25). In *Escherichia coli*, with a *recBCD* and *sbcBC* deletion background, RecJ and RecQ help to create 3' ssDNA overhangs (26–28). For bacteria that do not have RecBCD/AddAB enzyme family members, proteins such as RecJ, RecQ, UvrD, HelD, and the SbcC-SbcD complex are potential alterna-

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tive enzymes in 3'-end resection (29). Bioinformatical analysis indicted some bacterial lineages, such as the *Deinococcus-Thermus* phylum, also have NurA and HerA proteins with functions that have not yet been experimentally characterized (18).

Deinococcaceae, an extremely radioresistant bacterial family, has robust DSB repair capacity (30–33). These bacteria use the RecFOR pathway as the major recombinational DNA repair pathway and naturally lack RecBCD/AddAB systems (31, 34). In *Deinococcus radiodurans*, RecJ has 5'-to-3' ssDNA exonuclease activity (35). However, knocking out *recJ* only modestly decreases radioresistance, indicating that another nuclease might be involved in 3'-end processing in HR (35). Although RecQ, HelD, and RecD2 all possess DNA helicase activity (36–38), none of them makes a remarkable contribution to robust DNA repair in *D. radiodurans* (37, 39, 40). Cells lacking the helicase UvrD are more sensitive to gamma irradiation but are not comparable to *recA*, *recF*, *recO*, or *recR* mutants (39). Therefore, how *D. radiodurans* processes 3'-end DNA during HR is unknown. A *nurA-herA* operon is found in *D. radiodurans* (18). Both NurA and HerA are essential in archaea and important in DSB repair (41, 42). Therefore, their homologs in *D. radiodurans* are potential candidates for 3'-end-processing enzymes during HR.

We performed bioinformatical analysis of the *nurA-herA* operon and its expressed products in *D. radiodurans*. For biochemical studies, several site-directed mutations were made in conserved functional sites. We found that DrHerA had ATPase activity that was greatly enhanced by DrNurA or DNA, and DrNurA was a HerA-dependent manganese-preferring 5'-to-3' ssDNA/dsDNA exonuclease/endonuclease. DrHerA and DrNurA showed costimulation of activity that depended on direct physical interaction. We disrupted each gene separately or the two genes together in *D. radiodurans*. The mutants exhibited abnormal cell proliferation and decreased intermolecular recombination efficiency, indicating that these proteins might be involved in cell proliferation and recombinational DNA repair in *D. radiodurans*.

MATERIALS AND METHODS

Multiple protein alignment and structure modeling. The sequences of NurA and HerA proteins from *D. radiodurans*, *Sulfolobus solfataricus*, *Pyrococcus furiosus*, *Thermotoga maritima*, and other species were obtained from the NCBI website. Motif and active site predictions of HerA were based on an earlier studies of HerA from *Methanobacter thermoautotrophicus* (17), *Sulfolobus acidocaldarius* (16), *P. furiosus* (13), and *S. solfataricus* (43) and the TrwB protein (EcoTrwB) and FtsK protein (EcoFtsK) from *E. coli* (44, 45). The DrHerA three-dimensional structure was modeled based on the *S. solfataricus* HerA (SsoHerA) structure (PDB 4D2I) by using the Phyre2 online program (<http://www.sbg.bio.ic.ac.uk/phyre2>) (46). Motif and active site predictions of NurA were based on earlier studies of *P. furiosus* NurA (PfuNurA; PDB 3TAI) (13, 14) and *S. solfataricus* NurA (SsoNurA; PDB 2Y GK) (43). Multiple alignments of HerA and NurA were performed by using the Cobalt constraint-based multiple protein alignment tool on the NCBI website (<http://www.ncbi.nlm.nih.gov/tools/cobalt>), followed by manual corrections.

Strains, media, and transformations. All bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. All primers and oligonucleotides used in this study are listed in Table S2 in the supplemental material. All *E. coli* strains were grown in LB medium (1% tryptone, 0.5% yeast extract, and 1% sodium chloride) at 37°C; the medium was supplemented with the appropriate antibiotics (ampicillin, 100 µg/ml; kanamycin, 40 µg/ml).

All *D. radiodurans* strains were grown at 30°C in TGY medium (0.5% tryptone, 0.1% glucose, and 0.3% yeast extract). To transform *D. radio-*

durans cells, a modified CaCl₂ technique described previously was utilized (47). *D. radiodurans* cells were cultivated to the early stationary phase (optical density [OD], 0.8 to 1.0). The cells were harvested, washed twice with 2× TGY medium (with 30 mM CaCl₂), and resuspended in 500 µl 2× TGY medium (with 30 mM CaCl₂) and incubated at 30°C for 1 h. Aliquots containing plasmid or DNA fragments (200 ng) were added and incubated on ice for an additional 1 h. Five milliliters of fresh TGY medium was added to the aliquot, and the mixture was continuously incubated at 30°C for 24 h. Selection for *D. radiodurans* transformants was achieved on TGY plates supplemented with the appropriate antibiotics (chloramphenicol, 4 µg/ml; streptomycin, 8 µg/ml; kanamycin, 10 µg/ml).

All *Saccharomyces cerevisiae* AH109 strains were grown at 30°C in YPDA medium (1% tryptone, 2% glucose, 0.5% yeast extract, and 0.003% adenine). SD/-Leu/-Trp or SD/-Ade/-His/-Leu/-Trp medium (Clontech) was used as an alternative medium when needed.

Protein expression and purification. The full-length *D. radiodurans* *herA* (*drherA*; *dr_0837*) and *drnurA* (*dr_0836*) genes and the *drherA-C* fragment (*drherA* with deletion of the N-terminal HAS domain [135 amino acids]) were amplified by PCR from *D. radiodurans* genomic DNA and cloned into the NdeI and BamHI sites of the pET28b-HMT vector (48). The resulting expression vectors contained an HMT tag (6×His tag, maltose binding protein [MBP], and tobacco etch virus protease [TEV] cleavage site sequences) at the N terminals of these genes. Site mutations of *drherA* (K175A, D487N/E488Q, R495A, and R513A) and *drnurA* (D53A, E155A, D184A, K201A, and E321A) were introduced by using a site-directed mutagenesis kit (Stratagene, USA).

Wild-type, site-mutated, or HAS domain-deleted HerA and NurA were expressed in *E. coli* BL21(DE3)(pLysS) cells at 30°C for 5 h with induction by 0.5 mM isopropyl-β-D-thiogalactopyranoside when the OD at 600 nm (OD₆₀₀) reached 0.8. The cells were resuspended in lysis buffer A (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 10% [vol/vol] glycerol, and 1 mM β-mercaptoethanol) containing protease inhibitor cocktail (Roche Biochemicals, Switzerland) and lysed by sonication. After centrifugation at 15,000 × g for 40 min, the supernatant was loaded onto a Ni-nitrilotriacetic acid (NTA) column (GE Healthcare, USA), which was pre-equilibrated with lysis buffer. Target protein was eluted with elution buffer B (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 300 mM imidazole). The collected fraction was digested with TEV protease at 4°C overnight. An amylose column was used to remove the HMT tag. Then, the protein was dialyzed against buffer C (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 10% [vol/vol] glycerol, 1 mM EDTA, and 1 mM DL-dithiothreitol [DTT]), and loaded onto a Hitrap Q ion exchange column (GE Healthcare). Target protein was eluted by gradient elution. Finally, proteins were further purified by using a Superdex 200 (or 75) column (GE Healthcare) with 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10% glycerol, 1 mM DTT, and 1 mM EDTA. Fractions containing the target proteins were pooled, concentrated, and flash-frozen in liquid nitrogen and stored at −80°C. The purification procedures for wild-type and mutated NurA were almost the same as those for HerA.

For pulldown assays, the *nurA* gene was ligated into the pET28a expression vector to express NurA with an N-terminal 6×His tag. Protein was purified via Ni-NTA (GE Healthcare) affinity chromatography, Hitrap Q (GE Healthcare) ion exchange chromatography, and Superdex 75 (GE Healthcare) chromatography. The purity of each protein was checked by using silver stain and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Yeast two-hybrid assays. Yeast two-hybrid assays were carried out by following the protocol from the Matchmaker Gold Yeast two-hybrid system user manual (Clontech, USA). *drherA*, *drherA-C*, and *drnurA* genes were cloned into the NdeI and BamHI sites of the pGBKT7 and pGADT7 vectors and named BDH, BDHC, and ADN, respectively. BDH, BDHC, and ADN were transformed into AH109 together, and the transformants were selected on SD/-Leu/-Trp agar plates. In the meantime, pGBKT7 (BD) and pGADT7 (AD) vectors were also transformed as controls. The interaction was tested on SD/-Ade/-His/-Leu/-Trp agar plates with 3 mM

3-amino-1,2,4-triazole (3AT) and 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside (X- α -Gal).

Pulldown assays. Pulldown assays were performed as previously described with some modification (49). A 200- μ l volume of His-NurA (0.5 mM) was incubated with 20 μ l Ni-NTA-agarose beads (GE) and washed with washing buffer (100 mM NaCl, 20 mM Tris-HCl [pH 7.5], 0.05% Tween 20) three times and then incubated with 400 μ l 0.5 mM HerA or 400 μ l 0.5 mM HerA-C (with lysozyme as a control) at 4°C for 3 h. The beads were washed with washing buffer a few times so that the lysozyme was completely washed off. The proteins were eluted with 50 μ l elution buffer (500 mM imidazole, 100 mM NaCl, 20 mM Tris-HCl [pH 7.5]) and analyzed by 12% SDS-PAGE.

Analytical gel filtration. Physical interactions between HerA (wild type and C-terminal domain) and NurA were investigated by using analytical gel filtration as previously described with some modification (43). HerA alone (6 μ mol; protomer), NurA alone (3 μ mol; protomer), HerA-C alone (6 μ mol; protomer), or HerA (6 μ mol; protomer) and NurA (3 μ mol; protomer), HerA-C (6 μ mol; protomer), and NurA (3 μ mol; protomer) were mixed and preincubated at 30°C for 20 min in a total 300 μ l of gel filtration buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 5% glycerol, 1 mM DTT, 1 mM EDTA). Reaction mixtures were then spun at full speed at 15,000 $\times$ g for 5 min to remove any precipitated protein and loaded onto a Superdex S200 HR 10/300 (GE Healthcare) analytical gel filtration column. Fractions (0.5 ml) were collected and analyzed via 12% SDS-PAGE. The sizes of calibration proteins at the positions where they eluted were marked on the x axis, based on the method described for the gel filtration calibration kit HMW (GE Healthcare). Ovalbumin (43 kDa), conalbumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa) were used as size markers.

ATPase assay. An ATPase assay was previously described (45), which was measured by spectrophotometric detection of inorganic phosphate (P_i) with malachite green reagent (1:1:2:2, ammonium molybdate [5.72%, wt/vol], HCl [6 N], polyvinyl alcohol [2.32%, wt/vol], and malachite green [0.08712%, wt/vol], all in distilled water). Reaction mixtures (20 μ l) containing different proteins (1 μ M NurA protomer or 3 μ M HerA protomer), ATP (1 mM), $MgCl_2$ (1 mM), and different DNA substrates (10 μ M; using oligonucleotide 4 [O4] as the ssDNA substrate and O3 annealed with O4 as the dsDNA substrate), were mixed in the reaction buffer and incubated at 37°C for 20 min. (For temperature-dependent assays, different temperatures of 35, 40, 45, 50, 55, and 60°C were used.) Malachite green reagent (200 ml in 700 ml water) was added, and P_i release was monitored at 630 nm. The negative-control experiment was performed in the absence of HerA, NurA, and DNA, and this result was normalized to zero as a background measure. Standard P_i agent (0.02 mM) was included as a positive control. ATPase activity was analyzed by P_i release measurement, which can be calculated by the following equation: ATPase activity (free P_i released per HerA monomer) = $(OD_{630m}/OD_{630p}) \times [0.02/(3 \times 10^3)]$, where OD_{630m} represents values of different samples and OD_{630p} represents the value of the positive control. Nucleic acid blanks were included to ensure that P_i release was mainly from ATP. Substrate blanks at higher temperatures were also included to exclude the possibilities that ATP is vulnerable to higher temperatures and/or that malachite gives a false-positive reading at higher temperatures.

Nuclease activity assay. 5'-6-Carboxyfluorescein (FAM) and 3'-FAM fluorescence-labeled oligonucleotides O1 and O2 were synthesized by Sangon Biotec (China) and purified via PAGE. dsDNA substrates were obtained by annealing O1 or O2 with O3 (dsDNA), O5 (5'-overhang DNA substrate), or O6 (3'-overhang DNA substrate). Hairpin structure DNA substrates with overhangs or blunt ends were obtained by annealing O7 (5'-FAM fluorescence-labeled 5'-overhang hairpin structure), O8 (3'-FAM fluorescence-labeled 5'-overhang hairpin structure), O9 (3'-FAM fluorescence-labeled 3'-overhang hairpin structure), O10 (5'-FAM fluorescence-labeled blunt-ended hairpin structure), or O11 (3'-FAM fluorescence-labeled blunt-ended hairpin structure).

Reaction mixtures containing DrNurA (500 nM; protomer) with or without various concentrations of DrHerA (500 and 1,500 nM; protomer) and 50 nM substrate DNA in reaction buffer (25 mM Tris-HCl [pH 7.5], 60 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 10 mM $MnCl_2$ [or, for metal-dependent assays, 10 mM $MgCl_2$, $MnCl_2$, $CaCl_2$, and EDTA], and 0.1 mg/ml bovine serum albumin [BSA]) were incubated at 37°C (for temperature-dependent assays, temperatures of 35, 40, 45, 50, 55, and 60°C were used) for 30 min. The reaction was stopped by adding the same volume of 2 $\times$ reaction stop buffer (95% formamide, 50 mM EDTA, 0.05% SDS, 0.01% bromophenol blue), followed by boiling at 100°C for 5 min and flash-cooling on ice for 10 min. When ATP was needed, different concentration (0.25, 0.5, 1.0, and 2.0 mM) of ATP were added into the reaction system.

Reaction products were analyzed on 15% denaturing polyacrylamide gels containing 7 M urea in Tris-borate-EDTA (TBE) buffer. Gels were imaged within fluorescence mode (FAM) on a Typhoon FLA 9500 apparatus (GE Healthcare), and bands were analyzed by using Image J software (National Institutes of Health, USA), if necessary.

For nicking endonuclease activity, 50 ng/ μ l supercoiled plasmid DNA pUC18 (TaKaRa) was incubated with NurA (1 μ M) or HerA (3 μ M) in 10 μ l reaction buffer (25 mM Tris-HCl [pH 7.5], 60 mM KCl, 1 mM DTT, 10 mM $MnCl_2$ [for metal-dependent assays, 10 mM $MgCl_2$, $MnCl_2$, or $CaCl_2$, or EDTA were used] and 0.1 mg/ml BSA) at 37°C for 30 min. Reactions were stopped by 10 $\times$ SDS DNA loading buffer (50 mM EDTA, 5% SDS, 50% glycerol, and 0.05% bromophenol blue). The reaction solutions were then loaded onto a 1.0% agarose gel running in 1 $\times$ TBE buffer. Gel was stained with 0.5 μ g/ml ethidium bromide and visualized under UV light.

Mutant strain construction and complementation. Knockout of *nurA*, *herA*, or the *nurA-herA* operon was carried out using a deletion replacement method described previously (35, 50). The upstream and downstream target genes with BamHI and HindIII digestion sites, respectively, were amplified by PCR. After BamHI and HindIII enzyme digestion, these segments were ligated to a kanamycin resistance cassette (Kan^r) (with BamHI and HindIII enzyme digestion as well) and transformed into the wild-type *D. radiodurans* strain R1. The mutant strains (named NM, HM, and NHM) were screened with kanamycin-containing TGY plates and confirmed by PCR product analysis and sequencing.

Western blotting. The wild type and *nurA*, *herA*, and *nurA-herA* mutants were harvested when the cell density of the culture (OD_{600}) reached 1.0. Cells were washed and lysed in lysis buffer A, described above, with sonication on ice. Proteins were separated on 12% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (Millipore, USA). Rabbit anti-NurA polyclonal antibody (generated by our laboratory) and rabbit anti-HerA polyclonal antibody (generated by our laboratory) were applied to measure the expression levels of DrNurA and DrHerA by each strain, respectively. Rabbit anti-GroEL polyclonal antibodies (Sigma, USA) were used to measure the expression level of GroEL as a control. Horseradish peroxidase-conjugated goat anti-rabbit antibody (Beyotime Biotechnology, China) was used as a secondary antibody, and signal was detected with the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

Growth curve assay. Growth curve and temperature sensitive assays were performed as previously described (35). Briefly, after the cell density of the culture (OD_{600}) reached 1.0, 1-ml aliquots were resuspended in 100 ml new fresh TGY medium and incubated at 30°C or 37°C. The cell growth rate was monitored by measuring the OD_{600} after various incubation times. Three replicates were performed for each strain. The number of CFU per milliliter was also measured every 5 h during the growth assays. Growth curves were created by using GraphPad Prism 5.0.

DNA-damaging agent survival rate assays. Survival rate assays were performed as previously described (49). Cells were grown in TGY medium with the appropriate antibiotics to early exponential phase (OD_{600} , 0.6 to 0.8). For gamma irradiation, cells were irradiated on ice under different doses (0, 4, 8, 12, and 16 kGy) and then diluted to appropriate

FIG 1 The localization of *herA* and *nurA*. (A) Genomic organization of the *herA*, *nurA*, *mre11*, and *rad50* genes from representative species in archaea. *Afu*, *Archaeoglobus fulgidus*; *Pfu*, *Pyrococcus furiosus*; *Sso*, *Sulfolobus solfataricus*; *Pab*, *Pyrococcus abyssi*; *Pho*, *Pyrococcus horikoshii*; *Tac*, *Thermoplasma acidophilum*; *Tvo*, *Thermoplasma volcanium*; *Ape*, *Aeropyrum pernix*; *Mth*, *Methanobacter thermoautotrophicus*. (B) Genomic organization of the *herA*, *nurA*, *sbcD*, and *sbcC* genes from representative species in bacteria. *Mph*, *Micrococcus phosphovorus*; *Tcu*, *Thermomonospora curvata*; *Ams*, *Actinoplanes missouriensis*; *Nmu*, *Nakamurella multipartite*; *Mma*, *Mycobacterium marinum*; *Calo*, *Calothrix* sp.; *Ico*, *Ilumatobacter coccineus*; *Cpr*, *Coprothermobacter proteolyticus*; *Dra*, *Deinococcus radiodurans*; *Tth*, *Thermus thermophilus*; *Tra*, *Truepera radiovictrix*; *Msi*, *Meiothermus silvanus*; *Aae*, *Aquifex aeolicus*; *Tma*, *Thermotoga maritima*; *Bha*, *Bacillus halodurans*; *Chl*, *Chloroflexus* sp. Each operon structure has an added promoter and is separated by a slash.

concentrations and plated on TGY medium. For UV treatment, cells were first diluted to appropriate concentrations and plated on the TGY plates. After complete absorption, the plates were exposed to UV under different dose from 0 to 1,000 J/m² followed by plating on TGY plates. Three replicates were performed for each strain. Colonies were counted after culture at 30°C for 2 to 3 days.

Transformation efficiency assays. The transformation efficiency assays were carried out as previously described with some modifications (51). The shuttle vector pRADK, which has chloramphenicol resistance via a *cat* cassette, was transformed into the wild type and mutants (see the description of the transformation method described above). The final mixture was diluted appropriately with phosphate-buffered saline (PBS), plated onto either TGY agar plates or TGY agar plates supplemented with 4 µg/ml chloramphenicol, and incubated at 30°C for 3 days. The transformation efficiency was determined by calculating the number of transformant colonies that grew on chloramphenicol plates divided by the total number of viable cells that grew on TGY plates. Three replicates were performed for each strain.

Intermolecular recombination frequency assays. The recombination frequency assays were carried out as previously described, with some modifications (51). We isolated a rifampin-resistant strain (called RifR) carrying a site mutation (S438P) in RpoB, which is one of the common site mutations that occur spontaneously in the wild-type strain (52). A DNA fragment containing this mutation was amplified by PCR from RifR genomic DNA with primers PrifR-F and PrifR-R (see Table S2 in the supplemental material) and used to transform each strain. The final mixture was diluted appropriately with PBS and plated onto either TGY agar plates or TGY agar plates supplemented with rifampin (50 µg/ml) and incubated at 30°C for 3 days. The intermolecular recombination frequency was determined by calculating the number of rifampin-resistant colonies that grew on rifampin-containing plates divided by the total number of viable cells. The spontaneous mutation rate was also measured for each strain as a control. Three replicates were performed for each strain.

RESULTS

The *D. radiodurans* *nurA-herA* operon. All archaea and a few bacterial lineages have *nurA* and *herA* genes, which have not yet been detected in any sequenced eukaryotic genomes. In most archaea, *nurA* and *herA* genes are located in an operon containing *mre11* and *rad50*, with the gene order *herA mre11 rad50 nurA*, *mre11 rad50 nurA herA*, or *nurA herA mre11 rad50* (Fig. 1A). Cotranscription of these four genes was confirmed in *S. acidocaldarius* in reverse transcription-PCR assays (16). Also, in *Methanothermobacter* spp., the *herA* gene in the operon is split into two genes and the part encoding the C-terminal half is fused to the gene *mre11* (Fig. 1A).

In bacteria that contain *nurA* and *herA* genes, *sbcD-sbcC*, the ortholog of *mre11-rad50*, is usually located nearby (Fig. 1B). However, bacteria from the *Deinococcus-Thermus* phylum have separate *nurA-herA* and *sbcD-sbcC* operons (Fig. 1B). In *Thermotoga maritima*, *Aquifex aeolicus*, and *Bacillus halodurans*, the *nurA* and *herA* genes are not together in an operon (Fig. 1B). In *Chloroflexus*, the NurA homolog is encoded by a gene that is adjacent to the gene encoding a stand-alone HerA HAS barrel domain (Fig. 1B). In *D. radiodurans*, *dr_0836* and *dr_0837*, which encode the putative NurA and HerA proteins, respectively, are located in a single operon.

Active site predictions for DrHerA and DrNurA. Sequence alignments of DrHerA and other RecA-like ATPases (*SsoHerA*, *PfuHerA*, *EcoTrwB*, and *EcoFtsK*) showed that *D. radiodurans* HerA possesses conserved putative Walker A, Walker B, and sensor-1 domains, which have potential ATPase activity (Fig. 2A). To analyze the biochemical function of DrHerA, we identified several

A

B

C

FIG 2 Functional site predictions of DrHerA and DrNurA. (A) Sequence alignments of Walker A, Walker B, and Sensor-1 domains. Dr, *D. radiodurans*; Tm, *Thermotoga maritima*; Pfu, *Pyrococcus furiosus*; Sso, *Sulfolobus solfataricus*; Eco, *Escherichia coli*. Identically conserved residues are shown in white letters and partially conserved residue are shown in red letters. The conserved ATP binding site, ATP hydrolysis sites, DNA binding site, and arginine finger are marked with arrows. (B) Comparison of the side views of the SsoHerA protomer (PDB 4D21) and predicted DrHerA protomer. The HAS barrel domain, RecA-like ATPase domain, helix-bundle, and C-terminal brace are shown in blue, pink, green, and red, respectively. The extra flexible loop of the predicted DrHerA structure is shown in yellow. The bound AMP-PNP is represented by the colored stick. The basic loop residues (K363 of SsoHerA and R495 of DrHerA) are represented by sticks as well. (C) Sequence alignments of putative active sites on different NurAs. Dr, *D. radiodurans*; Dg, *Deinococcus geothermalis*; Tth, *Thermus thermophilus*; Tm, *Thermotoga maritima*; Pfu, *Pyrococcus furiosus*; Sso, *Sulfolobus solfataricus*. Identically conserved residue are shown in white letters, and partially conserved residue are shown in red letters. The conserved active sites and DNA binding site are marked with arrows.

key conserved residues by using published structural and biochemical information (53).

Amino acid sequence alignment using far evolutionary distance-originated proteins was not an accurate prediction method, so we built a homology DrHerA model based on the SsoHerA structure (PDB 4D21) (Phyre2 online program [46]) (53). By comparing the two HerA promoter structures, we found that a major difference between DrHerA and SsoHerA might be a longer helix bundle area and a less-structured flexible loop area in DrHerA (Fig. 2B), which make DrHerA nearly 100 amino acids longer than SsoHerA. The typical RecA-like ATPase domain and the HAS barrel domain in DrHerA appeared to be highly con-

served (Fig. 2B). The DNA binding residue of SsoHerA (K363) was a conserved arginine (R495) in DrHerA (Fig. 2B). Detailed information about the DrHerA ATP hydrolysis center is shown in Fig. S2 in the supplemental material. We hypothesized that DrHerA K175 (corresponding to SsoHerA K154) is a putative ATP binding site, D487 and E488 (corresponding to SsoHerA E355/E356) are putative ATP hydrolysis sites, and R513 (corresponding to SsoHerA R381) is a putative arginine finger. In SsoHerA, the conserved aspartate (D471) on the C-terminal brace forms a salt bridge with a *trans*-acting residue (R142) of the adjacent protomer that is critical for DNA-dependent ATPase activity. We were unable to find any acidic or basic residues in the corre-

sponding position of DrHerA. However, a conserved lysine (K607) on the C-terminal brace of DrHerA might form a salt bridge with a conserved glutamic acid (E159) near the active site of the adjacent protomer. Further alignments of bacterial HerA proteins showed substantial identities (see Fig. S1A in the supplemental material), especially for the putative functional sites described above.

All reported NurA structures contain an RNase H-like domain and show dimeric assembly. It has been shown that the channel formed by two protomers is too narrow to accommodate B-form dsDNA, but it might be suited to adjust one or two strands of an unwound duplex (14, 43). NurA secondary structure analysis suggests a low level of sequence similarity between paralogous and orthologous groups (18). Comparisons between three previously reported NurA structures indicated that NurA family members might not be highly structurally conserved. The only reported bacterial NurA structure from *T. maritima* (PDB 1ZUP) showed differences from other reported archaeal NurAs. Obvious differences were observed in the dimeric architecture into which the two N-terminal strands of TmNurA protrude to divide the central channel, while the PfuNurA (PDB 3TAI) and SsoNurA (PDB 2YGK) are not (see Fig. S3 in the supplemental material). The interface of the TmNurA dimerization seems less tight than the interfaces of PfuNurA and SsoNurA. This difference might have a negative effect on DNA digestion, since mutagenesis of the PfuNurA dimerization interface abolishes nuclease activities (14). Furthermore, the slightly acidic central channel of the TmNurA dimer seems less capable of binding DNA than the basic channel of PfuNurA and SsoNurA (see Fig. S3). Nevertheless, by comparing two other NurA structures, we determined that four of five residues (D49, E140, D157, and K177) located around the putative active site are conserved in all NurAs (Fig. 2E), and the E279 is conserved among most bacteria homologs (see Fig. S4 and S1B in the supplemental material). Therefore, one cannot rule out that TmNurA also possesses nuclease activity.

Despite length differences and low identity (<10%) between DrNurA and archaeal NurAs, we performed sequence alignment using bacterial NurAs (see Fig. S1B in the supplemental material). Based on the TmNurA structure and alignment results, we identified four conserved residues involved in the catalytic center, D53, E155, D184, and E321, and one conserved residue (K201) involved in DNA binding (Fig. 2C).

D. radiodurans NurA and HerA interact *in vivo* and *in vitro*. Although no direct interaction between HerA and NurA was reported for *S. acidocaldarius* (21), interactions have been confirmed for *S. solfataricus* (43, 53, 54) and *P. furiosus* (13). Based on the conserved operon clustering of NurA and HerA in *D. radiodurans*, we hypothesized that DrNurA and DrHerA might physically or functionally interact with each other.

We used yeast two-hybrid assays to investigate the interaction of the two proteins *in vivo*. NurA and HerA interacted with each other (Fig. 3A). This result was confirmed by the lack of interaction when the HAS domain, suggested to be critical for the *S. solfataricus* HerA-NurA interaction, was absent (Fig. 3A). Pull-down assays were used to investigate if the two proteins interacted *in vitro*. Full-length HerA and HAS domain-deleted HerA (HerA-C) were incubated with His-NurA on Ni-NTA beads. Large amounts of full-length HerA protein but not HerA-C were pulled down by His-NurA. This result indicated that HerA formed

stable complexes with NurA *in vitro* and that the HAS domain of HerA is essential for the interaction (Fig. 3B).

A previous *S. solfataricus* study showed that HerA and NurA interacted with a 6:2 stoichiometry (43), consistent with the crystal structure of the NurA-HerA complex (53, 54). We purified DrHerA and DrNurA separately for analysis by gel filtration chromatography (see Fig. S5 in the supplemental material). DrHerA alone (67.4 kDa per protomer) eluted at around 10 ml on Superdex 200 as hexamers (Fig. 3C), consistent with earlier reports for *M. thermoautotrophicus* (17) and *S. solfataricus* (43) and the SsoHerA structure (53, 54). DrNurA (38.3 kDa per protomer) alone eluted around 14 ml on Superdex 200, indicating a monomer-dimer equilibrium (Fig. 3C), in agreement with the dimeric NurA structures of *P. furiosus* (14) and *S. solfataricus* (43). When DrHerA and DrNurA were mixed at a 2:1 molar ratio, the proteins coeluted at an earlier volume (9.5 ml) than did DrHerA alone. However, excess DrNurA eluted around 14 ml on Superdex 200 (Fig. 3C), indicating that the two proteins formed a stable complex with a most likely stoichiometry of 6:2. Meanwhile, HerA-C (52.9 kDa per protomer) alone eluted as a hexamer around 11 ml on Superdex 200 but no longer formed complexes with NurA (Fig. 3C). The wider peak of the HerA-C hexamer compared to the HerA hexamer suggested a tendency to dissociate, indicating that deletion of the HAS domain might affect the stability of DrHerA polymerization.

DrHerA is an ATPase whose activity is enhanced by NurA or ssDNA/dsDNA. HerA is an ATP-dependent bipolar helicase in hyperthermophilic archaea (16, 17, 55). We found that DrHerA has ATPase activity, based on free- P_i release measurements. Mutation of the K175, R513, and D487/E488 conserved residues in ATP binding and ATP hydrolysis decreased ATPase activity (Fig. 4A). Both the wild type and a catalytically inactive mutant of NurA (D53A; discussed in the next section) enhanced HerA ATPase activity (Fig. 4A). Furthermore, both ssDNA and dsDNA enhanced HerA ATPase activity (Fig. 4A). Mutation of the predicted DNA binding site on HerA, at R495, no longer resulted in DNA stimulation, suggesting that R495 might play an important role in DNA binding. HerA lacking the HAS barrel still had ATPase activity and was stimulated by ssDNA/dsDNA but not by NurA.

All HerA ATPase activities in thermophilic archaea were identified at a high temperature (60 to 70°C), in accordance with their hyperthermophilic hosts. Given that most bacteria that possess *nurA* and *herA* genes are mesophilic or hyperthermophilic, we measured DrHerA ATPase activity at higher temperatures. DrHerA ATPase activity was temperature dependent, with an optimum temperature of 45°C (Fig. 4B), nearly 3-fold and 5-fold higher than the activities at the *D. radiodurans* optimum culture temperature (30°C) in single and complex forms, respectively. A thermal stability assay showed HerA and NurA are very thermally stable and that high temperatures (up to 60°C) cannot denature them (see Fig. S6 in the supplemental material).

DrNurA is a 5'-3' ssDNA/dsDNA exonuclease/endonuclease with activity stimulated by HerA. 5'-3' ssDNA/dsDNA exonuclease/endonuclease activities have been reported for *S. acidocaldarius* NurA (SacNurA) and *Sulfolobus tokodaii* NurA (StoNurA) (15, 56), while no nuclease activity is observed with SsoNurA or PfuNurA until HerA is added (13, 14, 43). To identify the biochemical functions of DrNurA, we carried out a series of nuclease activity assays. In the absence of DrHerA, DrNurA did not have

FIG 3 Interaction assay results for DrHerA and DrNurA. (A) Yeast two-hybrid assays for NurA-HerA interaction analysis. Yeast cells were transformed with both plasmid constructs (AD and BD constructs), and all transformed cells were plated on 2 dropout medium plates (SD/-Leu/-Trp). These cells were also patched on 4 dropout medium plates (SD/-Ade/-His/-Leu/-Trp) with 3 mM 3-AT and X-α-Gal to check whether the AD and BD gene products had strong interactions. (B) Pull-down assay results. A 400-μl aliquot of 0.5 mM HerA or HerA-C (with lysozyme as a control) was incubated with NurA and Ni-NTA beads at 4°C for 3 h and analyzed by 12% SDS-PAGE. Lane 1, His-NurA; lane 2, HerA plus lysozyme (input control); lane 3, HerA pulled down by His-NurA; lane 4, HerA-C plus lysozyme (input control); lane 5, HerA-C pulled down by His-NurA. (C) Analytical gel filtration results. (Left, top graph) HerA alone (6 μmol; protomer), NurA alone (3 μmol; protomer), HerA-C alone (6 μmol; protomer), or a mixture were analyzed with a Superdex S200 HR 10/300 column. Peaks of each injection were reconstructed by using GraphPad Prism software. (Left, bottom graph) Protein size standard curve created with ovalbumin (43 kDa), conalbumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa). (Right) Each eluted fraction was separated by 12% SDS-PAGE and analyzed via Western blotting.

nuclease activity on ssDNA or dsDNA (Fig. 5A). DrNurA nuclease activity against ssDNA/dsDNA was stimulated by DrHerA (Fig. 5A), and stimulation still occurred with the ATPase-inactive HerA mutant (K175 and D487/E488) (Fig. 5A). No ATP was added to the assay mixtures, suggesting that stimulation was independent of HerA ATPase activity. HerA-C showed no obvious stimulation of NurA (Fig. 5A), indicating that stimulation depends on the

physical interaction between DrHerA and DrNurA. These results were in agreement with the result in *S. solfataricus*, that SsoNurA is stimulated by an SsoHerA ATPase-inactive mutant (K154A) to digest the ssDNA region of a 5' overhang (43). However, this result contradicted the finding that an SsoHerA-inactive mutant no longer stimulated SsoNurA digestion of dsDNA (43). Thus, the NurA-HerA complex digested dsDNA in the absence of ATP, and

FIG 4 ATPase activity assays of HerA. (A) ATPase activity of HerA or HerA-NurA with different DNA substrates. The ATPase activities were measured for HerA, HerA-NurA, HerA-NurA (D53A), HerA (K175A)-NurA, HerA (D487N/E488Q)-NurA, HerA (R513A)-NurA, HerA (R495A), HerA (R495A)-NurA, HerA-C, and HerA-C-NurA (500 nM HerA hexamer or NurA dimer) in the absence or presence of 1 μ M ssDNA/dsDNA. Data shown are mean values for three independent experiments, and error bars depict the standard deviation (SD). (B) ATPase activity at different temperatures. ATPase activity (in micromolar per minute) was calculated after the HerA or HerA-NurA complex was incubated with ATP for 20 min at 30, 35, 40, 45, 50, 55, and 60°C.

the mechanism of HerA-dependent dsDNA digestion might differ between archaeal and bacterial NurA.

We compared nuclease activities against ssDNA, dsDNA, and dsDNA with either 5' overhangs or 3' overhangs under the same reaction conditions and in the absence of ATP. Since DrNurA alone showed no nuclease activity against ssDNA or dsDNA, we used the DrNurA-HerA complex in these assays. In order to exclude the possibility that incompletely annealing dsDNA would give rise to a false result, a series of 5'- or 3'-labeled 5' or 3' overhangs or blunt-ended hairpins were also used as substrates. All the results indicated DrNurA-HerA had higher nuclease activity on ssDNA than on dsDNA, and 5' ends were preferred (Fig. 5B). Furthermore, DrNurA showed HerA-independent nicking endonuclease activity against closed-circular DNA (see Fig. S7 in the supplemental material), implying that DrNurA follows different digestion models in the presence or absence of DrHerA.

Given that mesophilic features were seen for DrHerA ATPase activity, we also performed nuclease assays at 30, 35, 40, 45, 50, 55, and 60°C; the optimum reaction temperature was around 55°C (Fig. 5C). In addition, we tested the metal preference of NurA during nuclease activity by using three common divalent metals, Mg^{2+} , Mn^{2+} , and Ca^{2+} . Both endonuclease and exonuclease ac-

tivities were dependent on manganese ion (see Fig. S8 in the supplemental material).

ATP effects on ssDNA and dsDNA digestion. Since archaeal HerA enhances NurA-HerA dsDNA nuclease activity through an ATP-dependent translocase/helicase activity (53), we studied the effect of ATP on DNA digestion by DrNurA-DrHerA. Low concentrations of ATP (0.25 mM) enhanced DrNurA dsDNA exonuclease activity, but high concentrations (more than 1 mM) inhibited the reaction (Fig. 6A), and the blunt-ended hairpin substrate also showed similar results (see Fig. S9 in the supplemental material). Moreover, even low concentrations of ATP (0.25 mM) attenuated the ssDNA nuclease activity (Fig. 6B). In order to exclude the chelation effect of ATP, nuclease activities at high metal ion concentrations (0.5 to 20 mM) in the presence or absence of 0.5 mM ATP were compared (see Fig. S10 in the supplemental material). The result clearly showed that ATP could strongly inhibit the HerA-NurA complex nuclease activity, no matter what concentration of metal ion was used in the reaction mixture (see Fig. S10).

Site-directed mutation analysis of NurA-HerA nuclease activity. To further investigate the nuclease activity, we created site-directed mutations in both DrHerA and DrNurA. Mutations of key residues involved in catalysis (D53, E155, D184, K201, and

FIG 5 Nuclease activity of NurA. (A) NurA showed HerA-dependent 5'-3' ssDNA/dsDNA exonuclease activity. NurA nuclease activity on ssDNA/dsDNA was analyzed in the absence or presence of DrHerA, DrHerA-C, DrHerA (K175A), or DrHerA (D487A/E488A). (Left blot) ssDNA was the substrate; (right blot) dsDNA was the substrate. (B) Comparisons of nuclease activity on different DNA substrates. (Left) ssDNA, dsDNA, 5'-overhang dsDNA, and 3'-overhang dsDNA were used as the substrates at the same time we studied NurA-HerA substrate preference. (Right) Hairpin with 5'-overhang, 3'-overhang, or blunt-ended structure DNA was used as the substrate. The position where FAM label was found is marked with an asterisk. (C) Temperature preference assay results. Different temperatures (30, 35, 40, 45, 50, 55, and 60°C) were tested. (Left) The different nuclease activities were compared at different temperatures by using ssDNA as the substrate. (Right) The corresponding nuclease activity efficiencies were analyzed by using ImageJ software.

E321) reduced DrNurA nuclease activity to a remarkable extent (Fig. 7A), indicating that these sites are essential for activity. In the presence of 0.25 mM ATP, wild-type DrHerA showed stronger stimulation for dsDNA digestion, while the ATPase-inactive mutants of DrHerA (K175A, D487N/E488Q, and R513A) did not show such stimulation, indicating that the ATPase activity of HerA contributed to NurA dsDNA nuclease activity (Fig. 7B). With ssDNA digestion, however, ATPase-inactive DrHerA exhibited a similar stimulation degree of nuclease activity as the wild type, indicating that ATPase activity contributes little for ssDNA

digestion stimulation. Furthermore, the mutation of R495, which is believed to be involved in HerA DNA binding and influences HerA ATPase activity, had no effect on NurA dsDNA digestion but showed declined digestion on ssDNA. **HerA and NurA mutants result in abnormal proliferation.** Because both DrHerA and DrNurA showed mesophilic features *in vitro*, we studied the effects for HerA and NurA mutants growing at a normal culturing temperature (30°C) and a higher culturing temperature (37°C). *nurA*, *herA*, and the *nurA-herA* operon were knocked out by deletion replacement and verified by PCR (see Fig.

FIG 6 The effects of ATP on ssDNA/dsDNA digestion. (A) Low concentrations of ATP enhance dsDNA digestion. Different concentrations of ATP (0.25, 0.5, 1.0, and 2.0 mM) were added to the reaction mixture. (B) ATP attenuates ssDNA digestion. Different concentrations of ATP (0.25, 0.5, 1.0, and 2.0 mM) were added to the reaction mixture.

S11A to C in the supplemental material) and sequencing. To exclude the possibility that *nurA* knockout influenced transcription and expression of the downstream gene *herA*, we knocked out only the N-terminal 900 bp of *nurA* (1,050 bp total). Expression of

NurA and HerA in knockout mutants was confirmed by Western blotting (see Fig. S11D). The OD₆₀₀ value for each strain was measured hourly for 50 to 60 h (Fig. 8). Because the values for CFU per milliliter per OD₆₀₀ varied during the growth cycle, data for CFU

FIG 7 Effects of active site mutations on DrNurA and DrHerA. (A) Mutational analysis of effects of various DrNurA active sites on ssDNA digestion. Different NurA mutants (D53A, E155A, D184A, K201A, and E321A) were used to test the ssDNA nuclease activity with different ratios of DrHerA (1:1 or 1:3). (Top) Different nuclease activities, shown by different NurAs and using ssDNA as the substrate. (Bottom) The corresponding nuclease activity efficiencies were analyzed by using ImageJ software and are shown in the bar graph. (B) Mutational analysis of effects of DrHerA active sites on ssDNA/dsDNA digestion. The activities of wild type or different DrHerA site mutants (K175A, D487N/E488Q, R495A, and R513A) were compared in terms of ssDNA/dsDNA digestion. (Top) ssDNA was the substrate, and no ATP was added in the reaction mixture. (Bottom) dsDNA was the substrate and 0.25 mM ATP was added. D487N* stands for the D487N/E488Q double site mutant.

FIG 8 Growth curves of wild-type and mutant strains at different temperatures. The OD₆₀₀ values for the wild-type strain (R1), *nurA* disruptant (NM), *herA* disruptant (HM), and *nurA herA* double disruptant (NHM) were measured at different time points. The growth curves were created by using GraphPad Prism 5 software. Data shown are mean values for three independent experiments, and error bars depict the standard deviations (SD). (Left) Results for assay at 30°C; (right) results for assay at 37°C.

per milliliter were also measured every 5 h during the growth assays (see Fig. S12 in the supplemental material). Compared with the wild-type strain R1, all mutant strains showed a shorter growth cycle, with a shorter lag phase, shorter logarithmic phase, and shorter stationary phase, especially at 37°C (Fig. 8; see also Fig. S12). However, the saturated cell densities of mutants (OD₆₀₀, ≈5.5 at 30°C and ≈4.5 at 37°C) were much lower than that of the wild type (OD₆₀₀, ≈7.0 at 30°C and ≈5.9 at 37°C), indicating that mutants might be more vulnerable to the toxic metabolites. No obvious differences in cell size or morphology among these strains were observed. These phenotypes suggested that these two proteins might contribute to cell replication, metabolism, or apoptosis.

Deletion of HerA or NurA reduced the intermolecular recombination efficiency. To investigate the influence of HerA and NurA on intermolecular recombination, we used a Rif^r/RpoB system to compare intermolecular recombination efficiency among the wild type, *nurA*, *herA*, and *nurA-herA* disrupted strains. Since intermolecular recombination efficiency might be affected by transformation efficiency and spontaneous mutation frequency differs by strain, plasmid transformation efficiency and spontaneous Rif^r frequency were also tested. Differences in spontaneous mutation frequency were negligible among the strains (Table 1), so we calculated the relative intermolecular recombination efficiency, i.e., the intermolecular recombination frequency divided by the transformation efficiency. The *nurA*, *herA*, and *nurA-herA* deletion strains showed nearly 10-fold-lower relative intermolecular recombination efficiencies than the wild type (Table 1). Thus, the two proteins might be involved in intermolecular recombination. HerA belongs to the FtsK-HerA superfamily. FtsK has been reported to be critical for chromosome segregation as it activates recombination and decatenation and pumps chromosomal DNA across the septum (18). DrHerA-NurA might have a similar function in recombination activation. In addition, *nurA*, *herA*, and *nurA-herA* deletion strains had slightly higher plasmid transformation efficiencies than the wild-type strain. A possible explanation is that DrHerA-NurA is involved in resisting or pumping out exogenous nonhomologous DNA and therefore contributes to genome stability.

In archaea, both *nurA* and *herA* are upregulated after gamma irradiation treatment, indicating that these genes might contribute to a DNA DSB repair process (19, 20). We found that the two

genes were slightly upregulated after gamma irradiation treatment of *D. radiodurans* cells (data not shown); however, no significant reductions in gamma irradiation or UV irradiation resistance were observed in *nurA*-, *herA*-, or *nurA-herA*-disrupted strains (see Fig. S13 in the supplemental material).

DISCUSSION

In most archaeal genomes, the *nurA* and *herA* genes are in a conserved operon and work together. However, the NurA and HerA homologous proteins are present in only a few bacteria, such as *B. halodurans*, *Clostridium thermocellum*, *A. aeolicus*, and *D. radiodurans* (18). L. M. Iyer et al. reported that the bacterial *herA* gene might have emerged in archaea, followed by horizontal gene transfers to and between bacteria, providing a reconstruction of HerA/FtsK ATPase evolutionary history through phylogenetic analysis of the proteins themselves (18). They also mentioned that *herA* and *nurA* are usually located in the same operon and might have evolved together (18). We report here that *D. radiodurans*

TABLE 1 Intermolecular recombination, transformation, and spontaneous Rif^r mutation frequencies of *D. radiodurans* wild-type and mutant strains

Strain	Intermolecular recombination frequency (10 ⁻⁵) ^a	Transformation efficiency (10 ⁻⁶) ^b	Spontaneous Rif ^r frequency (10 ⁻⁸) ^c	Relative intermolecular recombination efficiency (10 ¹) ^d
R1	1.55 ± 0.25	0.45 ± 0.08	2.01 ± 0.12	3.44
NM	1.12 ± 0.15	1.921 ± 0.12	1.52 ± 0.18	0.58303
HM	1.02 ± 0.11	2.765 ± 0.17	1.02 ± 0.14	0.368897
NHM	1.09 ± 0.10	3.485 ± 0.094	1.24 ± 0.07	0.312769

^a The intermolecular recombination frequency is the number of Rif^r colonies divided by the total number of viable cells when *D. radiodurans* strains are transformed to Rif^r with 10 ng of donor DNA. Averages and standard deviations were calculated from three independent experiments.

^b The transformation efficiency is the number of transformant colonies divided by the introduced plasmid DNA (10 ng) and total number of viable cells. Averages and standard deviations were calculated from four independent experiments.

^c The spontaneous Rif^r frequency is the number of Rif^r colonies divided by the total number of viable cells without donor DNA. Averages and standard deviations were calculated from four independent experiments.

^d To allow for transformation efficiency, the relative intermolecular recombination efficiency of each strain was calculated as the intermolecular recombination frequency divided by the transformation efficiency.

NurA and HerA have activities that are conserved in relation to their archaeal homologs, including mutually dependent stimulation of activity and mesophilic features. These observations support the idea that bacterial *nurA* and *herA* were horizontally transferred from archaea as a gene pair.

It is interesting that in some archaea and bacteria, HerA is the proximal protein of the operon, while in other archaea and bacteria, such as *D. radiodurans*, HerA is not. Our *in vitro* results showed that HerA and NurA could interact with a 6:2 stoichiometry, in agreement with the interaction ratio for *S. solfataricus* (43, 53, 54). In addition, it seems that they form a functional complex with this stoichiometry, according to our ATPase and nuclease activity assays. However, it is usual that the distal protein of an operon would express less than the proximal one *in vivo*. Therefore, the possibility that DrNurA could function without DrHerA or have other partner proteins *in vivo* cannot be ruled out. It was reported that in *S. tokodaii*, NurA has physical and functional interaction with single-strand breaks (SSB) (56), suggesting that SSB proteins might be another candidate for NurA interaction *in vivo*. To our knowledge, proteins, such as SSB (RPA) and beta-clamp (PCNA), having multiple partners *in vivo*, usually participate in a number of processes. It is highly possible that distinct functions exist when NurA interacts with different partners *in vivo*.

Compared with SsoHerA (43), DrHerA has poor ATPase activity, which was 10- to 100-fold lower than the activity of SsoHerA. Moreover, in the absence of DNA, DrHerA alone had much lower ATPase activity than the DrHerA-NurA complex, while in the presence of DNA, the stimulation of NurA was not so obvious. Such a result is quite different from the result in *S. solfataricus* according to a previously reported study (43). As mentioned above, DrHerA might be a protein with a vast evolutionary distance from archaea, and the structures of SsoHerA and the putative DrHerA display some differences, indicating that their biochemical features might be different in detail. Also, low ATPase activity may explain the observation that dsDNA nuclease activity was far less efficient than ssDNA nuclease activity.

According to our data, the DrHerA protein could stimulate DrNurA nuclease activity on both ssDNA and dsDNA, while the ATPase activity of DrHerA only contributes nuclease digestion on dsDNA and not ssDNA. There are two possible explanations for this: in the absence of ATP, DrHerA enhances DrNurA activity through helping DrNurA binding for substrates; in the presence of ATP, HerA enhances DrNurA activity by playing the role of a translocase/helicase to provide more power for HerA-NurA complex movement along the DNA substrates. Given that the addition of ATP always reduced the digestion efficiency on ssDNA, we assumed the former pathway is the key reason of ssDNA nuclease activity stimulation. For dsDNA nuclease activity stimulation, both pathways should be taken into consideration. It has been reported that ATP binding to Rad50 induces a large structural change from an open form with accessible Mre11 nuclease sites into a closed form, leading loss of Mre11 nuclease activity and help regulate the DNA recognition and digestion process (57–59). It is also possible that ATP plays a regulation role for DrHerA-NurA activity *in vivo*.

In archaea, NurA and HerA, together with Mre11-Rad50, are important in DNA end resection during HR and might have a key role in DSB repair (13, 21). Lacking a RecBCD/AddAB system, *D. radiodurans* mainly uses the RecFOR pathway for DSB repair (31,

34). Deletion of potential nucleases or helicases in *D. radiodurans*, such as RecJ, sbcCD, RecD2, RecQ, UvrD, or HelD, causes only moderate radioresistance decline, suggesting that *D. radiodurans* has other nucleases and helicases for 3'-end resection (35, 37, 39, 40, 60). *nurA* or *herA* knockout strain showed reduced intermolecular recombination efficiencies but not significantly declined radioresistance. Therefore, how *D. radiodurans* performs 3'-overhang DNA end resection during HR is still unclear.

To our knowledge, archaea lack a clear homolog of the bacterial chromosome segregation protein FtsK, with no alternative protein reported yet (61). Given that HerA belongs to the HerA-FtsK superfamily and its DNA translocation activity has been confirmed, it is highly possible that HerA, in company with NurA, has multiple functions not only in DNA repair but also in chromosome segregation during cell division. This could explain why the *nurA* and *herA* genes are essential in archaea. However, both *herA* and *ftsK* genes are present in *D. radiodurans*, and *herA* appears to be nonessential. Moreover, we were unable to knock out the *ftsK* gene, indicating that the function of FtsK in *D. radiodurans* is nonsubstitutable.

It is worth noting that many species in the *Deinococcus-Thermus* phylum are mesophilic or hyperthermophilic. The optimum growth temperature of *D. radiodurans* is 30°C, which is 15 to 25°C lower than the optimum temperature for DrHerA and DrNurA activity. Therefore, it is quite possible that these two proteins have weak roles in *D. radiodurans* at the optimal growth temperature and that knocking out these genes results in only modest phenotypes. It is highly possible that after horizontal transfer from archaea, these two genes became silent or even redundant. Other alternatives could emerge in *D. radiodurans* to replace the original functions of these two genes.

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