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Mycoplasma gallisepticum Produces a Histone-like Protein That Recognizes Base Mismatches in DNA

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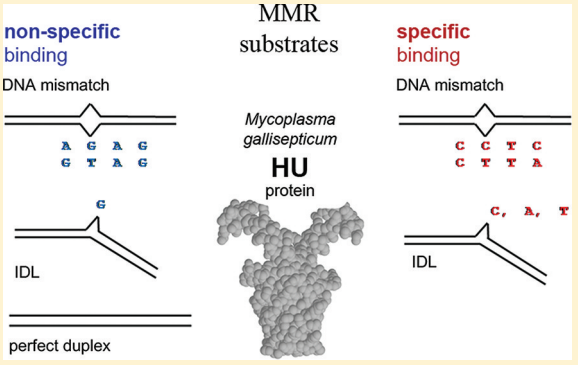
Supporting Information

ABSTRACT: Mycoplasmas are the smallest known microorganisms, with drastically reduced genome sizes. One of the essential biochemical pathways lost in mycoplasmas is methylation-mediated DNA repair (MMR), which is responsible for correction of base substitutions, insertions, and deletions in both bacteria and higher organisms. We found that the histone-like protein encoded by the *himA/hup_2* gene of *Mycoplasma gallisepticum* (mgHU) recognizes typical MMR substrates, in contrast to homologues from other species. The recognition of substitution mismatches is sequence-dependent, with affinities decreasing in the following order: CC > CT = TT > AA = AC. Insertions or deletions of one nucleotide are also specifically recognized with the following sequence-dependent preference: A = T > C. One-nucleotide lesions involving guanine are bound only weakly, and this binding is indistinguishable from binding to intact DNA. Although mgHU is dissimilar to *Escherichia coli* HU, expression in a slow-growing *hupAB E. coli* strain restores wild-type growth. The results indicate that mgHU executes all essential functions of bacterial architectural proteins. The origin and the possible role of enhanced specificity for typical MMR substrates are discussed.



In bacteria, the proper assembly of active higher-order genome structures requires accessory proteins. Nucleoid-associated proteins (NAPs), including LRP, FIS, H-NS, IHF, and HU, are involved in DNA supercoiling and compaction. They modulate vital DNA functions such as replication, recombination, repair, and transcription.[1−4] Each species is characterized by a specific set of NAPs, with only HU-like proteins being ubiquitous among bacteria. HU was first characterized as a histone-like protein in *Escherichia coli* because of its capacity to induce negative DNA supercoiling in the presence of topoisomerase I.[5] *E. coli* HU preferentially binds supercoiled DNA,[6,7] and cross talk exists between the relative amount of HU in the cell and the activity of topoisomerase I.[8] Later it was found that HU is also involved in DNA repair,[9,10] DNA recombination, and DNA replication.[11] Apparently, HU plays a pleiotropic role in bacteria; i.e., a single gene influences multiple phenotypic traits. A genome-wide screening showed that HU modulates transcription of 8% of *E. coli* genes, with only 10% of them possibly affected indirectly via supercoiling.[12] The functions and DNA binding properties of HU-like proteins may vary depending on the specific NAP content and the living conditions of the host.[13] Deletion of HU from the *E. coli* genome is not lethal unless IHF and H-NS are deleted as well.[14] In contrast, the absence of HU is lethal for organisms in which it is the only NAP available.[15,16]

In most bacteria, HU is a dimer of identical subunits, the heterodimeric HU being a characteristic of enterobacteria.[17] The crystal structure of the complex of HU with DNA was determined for *Anabaena* and *Borrelia burgdorferi*.[18,19] Two HU subunits are intertwined to form a compact α-helical body from which two long β-ribbon arms extend and interact with the DNA minor groove. Most of the HU-like proteins studied are characterized by weak nonspecific binding to dsDNA and much stronger and structure specific binding to distorted DNA structures such as forks, three- or four-way junctions, nicks, and overhangs.[20−23] The repertoires of preferred substrates vary greatly between the bacterial species.[13] *E. coli* HU binds dsDNA (B-form) like dsRNA (A-form) and DNA−RNA hybrids,[7,24] and it also avidly binds ssDNA.[25,26] It is not known, however, which of the properties listed above, if any, is relevant to the histone-like functions of HU.

Organisms belonging to the *Mycoplasma* genus (class Mollicutes characterized by the absence of the cell wall) represent

Received: June 13, 2011
Revised: August 22, 2011
Published: August 31, 2011

a branch of Gram-positive bacteria that emerged via reductive evolution accompanied by a partial loss of their ancestor's chromosomes.[27] These are the smallest-known microorganisms. They use a parasitic mode of life in a wide range of mammals, reptiles, fish, arthropods, and plants, with reduced biosynthetic capabilities compensated by the supply of host nutrients. The genomes of mycoplasmas are very small (from 0.58 to 1.4 Mb) and highly plastic.[28] A dozen complete genomes have been recovered, with one and sometimes two genes homologous to HU annotated (HimA/Hup_1 and HimA/Hup_2[29]). It was found also that Mycoplasma genomes involve none of the components of methylation-mediated DNA repair (MMR).[30] MMR is ubiquitous in organisms from bacteria to mammals and is responsible for correction of the most frequent genome lesions that appear in the course of DNA replication and homologue recombination, that is, base substitutions, insertions, and deletions.[31] This does not mean, however, that DNA repair is unnecessary for mycoplasmas because the specific enzymes for nucleotide/base excision and recombination repair pathways are kept.[30,32] Moreover, the mutation rates measured for mycoplasma phenotypes defined by resistance to specific antibiotics appear to be similar to the *E. coli* values.[33,34]

This study checks if small bacteria of the *Mycoplasma* genus possess proteins that are able to recognize DNA lesions commonly repaired by the MMR pathway. The poultry pathogen *Mycoplasma gallisepticum* is a small bacterium that causes chronic respiratory disease in chickens and infectious sinusitis in turkeys. The binding activity toward specifically damaged DNA was measured in cell extracts, and a single protein factor with relevant properties was found. It turned out to be the product of the HimA/Hup_2 (Swiss-Prot entry Q49504) gene earlier annotated as one of the two *E. coli* HU homologues available in the *M. gallisepticum* genome. This protein called here mgHU exhibits strong specific binding to mismatches, including single-nucleotide substitutions and insertions or deletions. Such lesions are recognized in other bacteria by the key MMR protein MutS rather than HU. Although the *E. coli* and *M. gallisepticum* HU proteins exhibit contrasting affinities and binding modes for undamaged DNA, the functional homology between them was confirmed by in vivo complementation tests. The expression of the second annotated HU homologue (HimA/Hup_1, Swiss-Prot entry Q7NBW7) was checked and confirmed. The corresponding protein probably has a different function. The results indicate that mgHU executes all essential functions of NAPs and that it is adapted to the absence of MMR in the mycoplasma genome. The functional role of the enhanced specificity for typical MMR substrates is unclear.

MATERIALS AND METHODS

DNA Sequences. A series of 48-mer dsDNA fragments with single-base mismatches in the middle was obtained from the sequence denoted J, CTGACCTAAGGGATC**CG**TCGTAGC**AA**<u>**GG**</u>ACTCAACTGCACTCTAGACT, and a complementary sequence denoted D, AGTCTAGAGTGCAGTTGAGT**C**CTTGCTACGACGGATCCCTTAGGTCAG. Sequence J was used in previous studies.[26] To obtain CA, CC, and CT mismatches, the italic and bold G residue in strand J was changed to A, C, and T, respectively. For all other possible single-base mismatches, the italic and bold complementary C residue in strand D was changed to A, G, and T, respectively. In the following text, the mismatches are denoted as XY, with residues X and Y corresponding strands D and J, respectively. The dsDNA fragments containing inserts of one, two, three, or seven unpaired nucleotides (bulges) were obtained by annealing the intact strand D with modified forms of strand J in which additional nucleotides were introduced between the two underlined G residues. Alternatively, to check the sequence context effects, the additional nucleotides were introduced into strand J between the bold CG and AA residues. These cases are indicated in the text.

The dsDNA fragments containing extended mismatches of two to four residues were obtained by annealing the intact strand D with modified forms of strand J with the following sequences (the mismatched bases are italic and bold): m2, CTGACCTAAGGGATCCGTCGTAGCAA***A***CACTCAACTGCACTCTAGACT; m3, CTGACCTAAGGGATCCGTCGTAGCAA***CG***CTCAACTGCACTCTAGACT; m4, CTGACCTAAGGGATCCGTCGTAGCAA***TGT***TCAACTGCACTCTAGACT.

The 5′-labeled oligonucleotide D was annealed with partially complementary nonlabeled oligonucleotides derived from sequence J, with the latter taken in triple molar excess. We conducted the annealing by incubating the oligonucleotides (3−12 μM) for 3 min at 90 °C in 20 mM Tris-HCl (pH 8.0) and 200 mM NaCl and then allowing them to cool slowly (∼4 h) to 40 °C.

***M. gallisepticum* Growth and Extraction.** *M. gallisepticum* strain L S6 was grown at 37 °C in medium containing (per liter) 20 g of tryptose (mixed enzymatically digested protein BioChemika), 5 g of NaCl, 3 g of Tris base, 1.3 g of KCl (pH 7.4, adjusted with HCl) supplemented with 1% glucose, 50 mL of liquid yeast extract, 100 mL of heat-inactivated (56 °C, 30 min) horse serum, and 200 units/mL penicillin. Bacteria were stored at −75 °C in the same medium. To obtain separated colonies, *M. gallisepticum* was grown in the same medium supplemented with 0.4% agar−agar. For protein extraction, the medium (4 mL) was inoculated with frozen bacteria and grown for 2 days. After that, 100−400 mL of the medium was inoculated with $^1/_{100}$ volume of this culture, further grown for 3 days, and harvested at 16000*g* (4 °C for 25 min). The pellet was washed three times with 20 mM Tris-HCl (pH 8), 150 mM NaCl, and 5 μg/mL PMSF and resuspended in 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, and 5 μg/mL PMSF to $^1/_{50}$ of the original volume of the growth medium. This suspension was subjected to three freeze−thaw cycles, with NP-40 added to a concentration of 0.24% after the first, NaCl added to a concentration of 800 mM after the second, and EDTA added to a concentration of 1 mM after the third cycle. After two additional freeze−thaw cycles, the suspension was centrifuged at 16000*g* (4 °C for 25 min). The supernatant was dialyzed against 10 mM Tris-HCl (pH 8), 0.1 mM EDTA, and 0.1 μg/mL PMSF, speed-concentrated to reduce the volume 5-fold, dialyzed again with the same buffer, and stored at −75 °C. This *M. gallisepticum* cell-free extract was used for gel mobility shift assays and protein identification.

Gel Mobility Shift Assays. Binding of *M. gallisepticum* proteins to DNA was tested by using band shift assays. A bound protein retards migration of DNA through the nondenaturing gel, which gives a shifted band. Varied amounts of *M. gallisepticum* extract protein (0.01−5 μL of extract) were incubated with 5′-labeled DNA (50 nM) for 15 min in 8 μL of binding buffer, 25 mM Tris-HCl (pH 8.0), 9% glycerol, and various NaCl concentrations. Samples were loaded onto nondenaturing 8% polyacrylamide gels (30:1) buffered with 100 mM (or 25 mM if indicated) Tris-borate and electrophoresed (555 V and 11 °C). A gel with 20 cm × 18 cm × 0.1 cm plates was prerun at 444 V for 2 h before samples were

loaded. The figures show representative results of 3−10 independent assays with each DNA construct.

Isolation of Protein for Identification by MALDI-MS. DNA mismatch-binding proteins were purified from the *M. gallisepticum* extract by using two-dimensional (2D) PAGE. Conditions of the first (nondenaturing) electrophoresis were as follows. Forty microliters of extract was mixed with 100 pmol of 5′-labeled DNA mismatch in 25 mM Tris-HCl (pH 8.0) and 9% glycerol and electrophoresed through 8% polyacrylamide gels (30:1) buffered with 100 mM Tris-borate (pH 8.3). The control gel was used under the same conditions without added DNA. The gel strips were soaked in 25 mM Tris-HCl (pH 8.0) and 0.2% SDS for 10 min and polymerized on the top of the stacking gel for the following SDS−PAGE [acrylamide gradient from 9 to 18% (30:1)]. The final gels were scanned and stained with silver to locate DNA and proteins, respectively. The relevant protein spots were subsequently cut out and subjected to trypsin in-gel hydrolysis.[35]

Identification of Protein by MALDI-MS. Aliquots (1 μL) of the sample were mixed on a steel target with 0.3 μL of a 2,5-dihydroxybenzoic acid (Aldrich) solution (20 mg/mL in 30% acetonitrile and 0.5% trifluoroacetic acid), and the droplet was left to dry at room temperature. The matrix-assisted laser desorption ionization (MALDI) mass spectra were recorded on an Ultraflex II MALDI-TOF-TOF mass spectrometer (Bruker Daltonik) equipped with a Nd laser. The $[MH]^+$ molecular ions were measured in reflector mode; the accuracy of monoisotopic mass peak measurements was within 50 ppm. After that, the Mascot (http://www.matrixscience.com) peptide fingerprint search was performed through the NCBI protein database. Oxidized methionine and propionamide cysteine were allowed as modifications. A candidate protein was considered significant if its score was greater than 80 ($p < 0.01$). A combined MS +MS/MS search with Biotools 3.0 software (Bruker Daltonik) further increased the score. Two proteins were identified as DNA binders, namely, gyrA (score of 90) and HimA/Hup_2 (score of 105). For the latter, three independent identifications were performed starting from independent extract preparations.

Evaluation of Dissociation Constants. We start with a sample containing a DNA binding protein and a DNA substrate that is able to form only one type of DNA−protein complex. The total protein concentration, P_o, is known, as well as the concentration of the added DNA, S_o. Using band shift assays, the relative intensities of the gel bands corresponding to bound and free DNA can be measured accurately. Their ratio (r = [bound]/[free]) can be used to evaluate the absolute concentrations of free and bound DNA, S_f and S_b, respectively, as

$$S_b = S_o r/(1 + r)$$

and

$$S_f = S_o/(1 + r)$$

By definition, the dissociation constant is

$$K_d = P_f S_f / S_b$$

where P_f denotes the free protein concentration. If the protein forms only specific 1:1 complexes with DNA, then

$$P_o = P_f + S_b$$

Via combination of these equations, the following relationship is derived

$$S_b = P_o - K_d r$$

Thus

$$K_d = P_o/r - S_o/(1 + r)$$
$$= P_o[\text{free}]/[\text{bound}] - S_o[\text{free}]/([\text{bound}] + [\text{free}])$$

with r obtained from a single band shift assay with the given DNA substrate. These relations are correct even if $S_o > K_d$; it is most important that the [free]/[bound] ratio be measured accurately.

The mgHU concentration in cell-free extracts of *M. gallisepticum* was evaluated by comparing the recombinant mgHU with that in the extract in a binding assay with the same reference substrate and assuming that the two proteins have similar binding constants. Additional discussion of the evaluation of dissociation constants from band shift assays can be found in ref 26.

Cloning, Expression, and Purification of His-Tagged *M. gallisepticum hup_2* from *E. coli*. For amplification of the *hup_2* gene of *M. gallisepticum*, the polymerase chain reaction (PCR) primers (forward, NdeI site underlined, atgcacatatgtttat-tatggcaaaaatcaaatc; reverse, BamHI site underlined, atgcggatccc-tatttgtgcgaatctac) were designed on the basis of the *hup_2* (Swiss-Prot entry Q49504) gene sequence obtained from the annotated genome data. *M. gallisepticum* genomic DNA was used as a template for the PCR. The PCR product was digested with NdeI and BamHI, ligated with the NdeI-BamHI-digested pET15b vector (Novagen), and transformed into *E. coli* DH5α cells. The nucleotide sequence was determined on both strands and was found to be the same for five clones. The recombinant plasmid was named pET15b-hin. For protein purification, the pET15b-hin plasmid was transformed into *E. coli* strain B834(DE3) (Life Technologies). The *E. coli* B834(DE3)/pET15b-hin mixture was then incubated in 300 mL of a LB medium containing 100 μg/mL ampicillin at 37 °C until the optical density at 600 nm reached ~0.8; IPTG was added to a final concentration of 0.1 mM to induce expression, and the cells were incubated for an additional 3 h. Cells were then disrupted by sonication, and the expressed hup_2 was purified using the HisTrap histidine-tagged protein purification kit (Amersham Biosciences) according to the manufacturer's protocol with minor modifications. Protein concentrations were measured using the Bradford method (Bio-Rad Laboratories).

The protein purity was estimated with an SDS gel to be >95%, and the protein migrates as 16 kDa polypeptide (Figure S1A of the Supporting Information). To further estimate the protein purity and verify its amino acid composition, we analyzed the purified protein by MALDI mass spectrometry without proteolytic digestion (Figure S1B of the Supporting Information). The molecular mass of the detected polypeptide was 13 386 Da. No traces of *E. coli* HU or other proteins were found.

Cloning and Expression of *M. gallisepticum hup_1* and *hup_2* Genes in *E. coli*. Genomic DNA of *M. gallisepticum* was purified with the genomic DNA purification kit (Promega) according to the manufacturer's protocol. Polymerase chain reactions were conducted with Pfu polymerase (Fermentas and Promega) for 30 cycles as follows: 94 °C for 1 min and 52 °C for 1 min and 72 °C for 5 min to amplify genes *hup_1* and *hup_2*. The following primers were used in the amplification: for *hup_2*, GGAATTCCATATGgcaaaaatcaaatcattaagtgctgctg and GCTCTA-GAgtattatataagatcttgattaactatttgtgc; for *hup_1*, GGAATTCCA-TATGatgctaactaaatctgaaatttgc and GCTCTAGAC-TAGCGGTTCTTAAAGAATCCAGCGGC. The resulting fragments corresponding to open reading frames *hup_1* and *hup_2* were gel purified, digested with NdeI and XbaI, and cloned in

expression vector pBADN[12] to generate plasmids pJO193 and pJO201, respectively. In each case, the expression of the cloned gene is under the control of the arabinose-inducible promoter of the *E. coli ara* operon.

RESULTS

***M. gallisepticum* Possesses Proteins That Recognize DNA Mismatches.** Band shift assays of cell-free extracts of *M. gallisepticum* with 5′-labeled dsDNA containing single-base mismatches revealed two major DNA binding protein components distinguished as two dense retarded bands in Figure 1. The upper band has a similar intensity for all DNA substrates regardless of the presence of mismatches. Such a pattern corresponds to nonspecific binding to intact dsDNA. In contrast, the second protein component responsible for the lower retarded band evidently distinguishes dsDNA with single mismatches in the middle and binds them more avidly than undamaged duplexes. This mismatch binding activity is sequence-dependent, with the CC mismatch-containing oligonucleotide showing the strongest affinity for the protein. The CC mismatch substrate was further used for identification of the detected DNA-binding proteins in cell-free extracts.

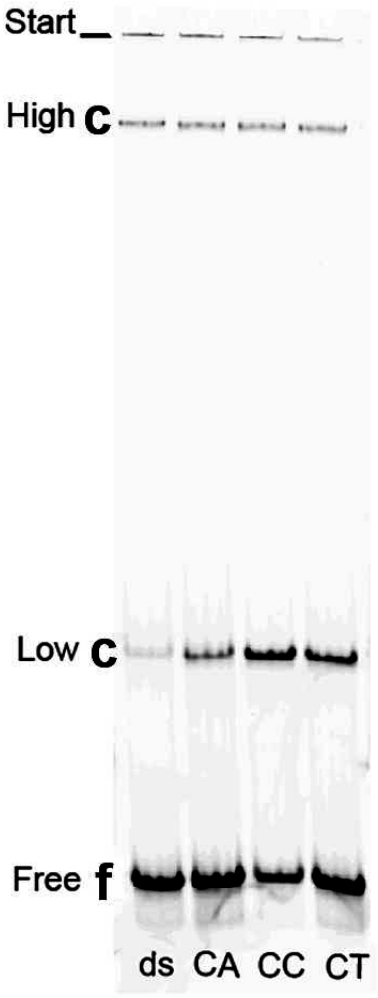


Figure 1. Testing DNA mismatch binding activity in cell-free extracts of *M. gallisepticum*. Protein–DNA complexes migrate in gels slower than free DNA and produce shifted bands. f and c mark the positions of free and complexed DNA, respectively. A 48 bp labeled oligonucleotide with a cytosine residue in the middle was annealed with a complementary oligonucleotide carrying guanine (lane ds), adenine (CA), cytosine (CC), and thymine (CT) bases at the corresponding position. These dsDNA fragments were incubated with *M. gallisepticum* extracts (40 mM NaCl), and the reaction mixtures were separated on a nondenaturing polyacrylamide gel.

Purification and Identification of the Mismatch-Binding Proteins of *M. gallisepticum*. Identification of protein components that recognize DNA mismatches was conducted by 2D gel purification combined with MALDI-TOF mass spectrometry. MALDI-MS is very sensitive (femtomolar range) and allows identification of proteins in silver-stained gels when the sample contains one or a few components. The results of these experiments are shown in Figure 2. The first electrophoresis of the *M. gallisepticum* extract was conducted under nondenaturing conditions at pH 8.3 (two top lanes in Figure 2). With this pH value, only negatively charged proteins with a pI of <8 can enter the gel. DNA binding proteins are commonly characterized by higher pI values and therefore should remain at the gel origin unless appropriate DNA substrates are added to the sample. In this case, the DNA (CC mismatch) substrate was added in the right lane only, and the gels were scanned for labeled DNA positioning. The left lane confirms the absence of DNA. The four bands in the right lane are attributed to free ssDNA, free dsDNA, a specific protein–DNA mismatch complex, and a nonspecific protein–dsDNA complex (from left to right, respectively).

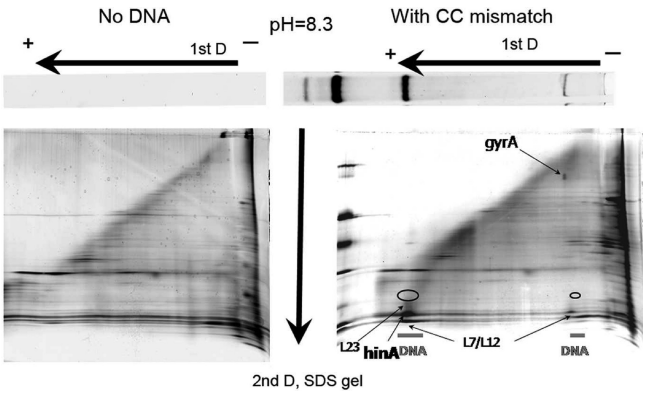


Figure 2. Analysis of mismatch-binding proteins of *M. gallisepticum* and their purification for MALDI identification. The *M. gallisepticum* extract in the absence of added DNA or with added CC mismatch (indicated) was loaded onto the 1D polyacrylamide gel and electrophoresed under nondenaturing conditions at pH 8.3; DNA was visualized (top), and gel strips were polymerized onto the origin of the perpendicular SDS–PAGE gel and the resultant gels scanned to detect DNA positions (indicated by ovals) and stained with silver to detect proteins (bottom). The spots indicated by arrows were cut from the gel, subjected to trypsin digestion, and analyzed with MALDI-MS.

The gel strips of the first electrophoresis were polymerized onto the origin of the perpendicular SDS–PAGE gel, and the resultant gels were stained with silver (two bottom panels of Figure 2). The locations of the DNA spots revealed by gel scanning are shown as ovals. Examination of the right 2D gel in Figure 2 reveals several spots of protein silver staining absent in the left gel that can be attributed to DNA binding. These spots as well as some other major spots corresponding to dsDNA bands in the first gel dimension were subjected to trypsin digestion followed by MALDI-MS. The spot corresponding to the uppermost DNA position in the one-dimensional (1D) gel was identified as the DNA gyrase A subunit. The spot

dx.doi.org/10.1021/bi2009097 | *Biochemistry* 2011, 50, 8692–8702

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                        1         10              20         30         40
                        |         |               |          |          |
consensus     -         *n*n***nn*nnnnn.....*n*nnnnnn*nn**nn*nnn*nn**n
mgHU          MAKIKS    LSAAEYLKEMADETN.....LKVQDIRLVVTSLQKVLAKELATTGE
hup_1         M         LTKSEICKIIAECTG.....VSPKLVKACFQVYSDLVKKEIKSQGQ
aclHU         -         MNKTELVALVADKAE.....VTQAMAEKVVNSFVDVVTETLSKDEK
bsuHU         -         MNKTELINAVAEASE.....LSKKDATKAVDSVFDTILDALKNGDK
TF1           -         MNKTELIKAIAQDTE.....LTQVSVSKMLASFEKITTETVAKGDK
ecoHUα        -         MNKTQLIDVIAEKAE.....LSKTQAKAALESTLAAITESLKEGDA
ecoHUβ        -         MNKSQLIDKIAAGAD.....ISKAAAGRALDAIIASVTESLKEGDD
T.maritima    -         MTKKELIDRVAKKAG.....AKKKDVKLILDTILETITEALAKGEK
Hbb           MSFSRRPK  VTKSDIVDQIALNIKNNNLKLEKKYIRLVIDAFFEELKSNLCSNNV
Anabaena      -         MNKGELVDAVAEKAS.....VTKKQADAVLTAALETIIEAVSSGDK

                       50         60         70         80         90
                       |          |          |          |          |
consensus     *n*n***n*n*nn*nn*n.n*******nn*n*nnnnnnn**n**n**nn*
mgHU          VRLFDIGKFKLVATKPRT.GINPKTKQKIQIPAGKKIKLTVSKILTDAVDSHK
hup_1         VRLPELGTFRVTIGRERI.SVNPITGAQTRIPPKPKVKFRAAKPLKEVTATIKWKYVSEDDELLQPKRKATVFFKNR
aclHU         VVVTGFGTFEVRNRVARR.GKNPRTGEEIIVPAQKTPAFKAGKLLKDAVK
bsuHU         IQLIGFGNFEVRERSARK.GRNPQTGEEIEIPASKVPAFKPGKALKDAVAGK
TF1           VQLTGFLNIKPVARQARK.GFNPQTQEALEIAPSVGVSVKPGESLKKAAEGLKYEDFAK
ecoHUα        VQLVGFGTFKVNHRAERT.GRNPQTGKEIKIAAANVPAFVSGKALKDAVK
ecoHUβ        VALVGFGTFAVKERAART.GRNPQTGKEITIAAAKVPSFRAGKALKDAVN
T.maritima    VQIVGFGSFEVRKAAARK.GVNPQTRKPITIPERKVPKFKPGKALKEKVK
Hbb           IEFRSFGTFEVRKRKGRLNARNPQTGEYVKVLDHHVAYFRPGKDLKERVWGIKG
Anabaena      VTLVGFGSFESRERKARE.GRNPKTNEKMEIPATRVPAFSAGKLFREKVAPP
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Figure 3. Multiple-sequence alignment of HU homologues. The residue numbering corresponds to that of *Bacillus subtilis* HU (bsuHU). In the consensus sequence, conserved and nonconserved residues are denoted with asterisks and the letter n, respectively. Dots indicate gaps in the alignement. HU homologues were from *M. gallisepticum* (mgHU and hup_1), *Acholeplasma laidlawii* (alcHU), *B. subtilis* (bsuHU), *B. subtilis* bacteriophage SPO1 (TF1), *E. coli* (ecoHU*α* and ecoHU*β*), *Thermotoga maritima*, *Borrelia burgdorferi* (Hbb), and *Anabaena*.

corresponding to the CC mismatch specific lower band in the 1D gel was identified as the HimA/Hup_2 (Swiss-Prot entry Q49504) gene product. *Hup_2* encodes a 9 kDa polypeptide earlier annotated as a homologue of the *E. coli* HU protein (cd00591 Sequence Cluster, HU_IHF).[29] The genome of *M. gallisepticum* contains one more such homologue (HimA/Hup_1, Swiss-Prot entry Q7NBW7) that encodes a larger 10 kDa polypeptide. Surprisingly, no peptides corresponding to HimA/Hup_1 were found either within the spot corresponding to the hup_2 protein or in the surrounding gel area. We conclude that protein HimA/Hup_1 does not bind DNA mismatches and also does not form heterodimers with HimA/Hup_2 capable of such binding. Hereafter, the HimA/Hup_2 protein is termed mgHU. Other proteins identified in the spots were ribosomal proteins L23 and L7/L12 (Figure 2). Supposedly, they bind DNA because of their high positive charges. In the cell, they should be incorporated into ribosomes and cannot be involved in interactions with DNA.

Figure 3 shows the sequence comparison of mgHU and the Hup_1 polypeptides with several homologous proteins from different species. A few features of the mgHU sequence are notable. The protein is moderately charged compared to canonical HU (+10 compared to +2 for bsuHU and +4 for ecoHU). A part of the excess charge comes from the MAKIKS N-terminal hexapeptide extension. Similar N-terminal tails are observed in homologous proteins of other Mollicutes, especially those belonging to Pneumoniae cluster (not shown). Both N- and C-terminal tails interact with DNA and can strongly affect the properties of HU-like proteins.[12] In contrast to mgHU, the *Hup_1* gene product involves a 24-residue C-terminal extension, suggesting that the two proteins have distinct functions.

Mismatch Binding by the Intact mgHU and by Recombinant His-Tagged mgHU. The specific mgHU binding to damaged DNA was confirmed by experiments with the purified recombinant His-tagged protein. The *M. gallisepticum HimA/Hup_2* gene was cloned and expressed in *E. coli*, and the corresponding polypeptide was purified as described in Materials and Methods. Binding to a series of dsDNA substrates carrying typical lesions is demonstrated in Figure 4. Panels A and B compare the band shift assays with *M. gallisepticum* extracts and the purified His-tagged mgHU, respectively. In both cases, the same set of DNA substrates was used. The patterns revealed in the two panels are similar. We conclude that the HimA/Hup_2 protein identified as a mismatch CC binder is also responsible for the binding to other DNA mismatches presented in Figure 4. A similar test with the *HimA/Hup_1* gene product did not reveal any DNA binding (see Figure 4A). dsDNAs with inserted A, C, or T (single-base IDLs) are efficiently and structure specifically bound by mgHU (Figure 4). The repair of these lesions is probably most important because they often lead to lethal frame shift mutations. Binding to single-base substitution mismatches is sequence-dependent, with pyrimidine-pyrimidine mismatches recognized more readily. On the basis of these results, we can identify mgHU as the specific mismatch-binding factor (revealed in experiments shown in Figures 1 and 2).

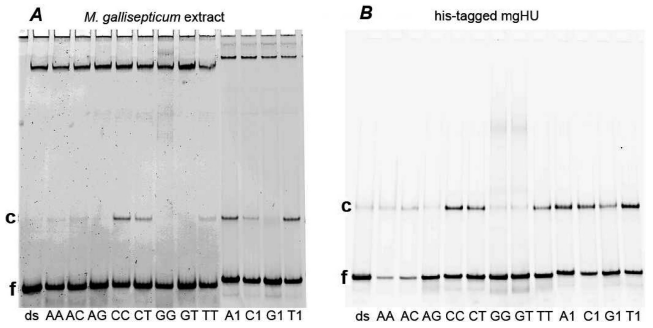

Figure 4. Comparison of DNA binding profiles observed for cell-free extracts of *M. gallisepticum* and the refined His-tagged mgHU in 100 mM Tris-borate gels. Panel A shows the binding pattern for the cell-free extracts of *M. gallisepticum* (0.25 μL per lane) with a series of dsDNA fragments carrying a single-base substitution or insertion mismatch in the middle. Labels A1, C1, G1, and T1 correspond to the inserts of the four corresponding bases. Panel B exhibits the binding pattern for the recombinant his-HU (2 μM) for the same DNA substrates as in panel A. All assays were performed at 150 mM NaCl. Other notations are similar to those in Figure 1.

By using *E. coli* HU as a reference, it was confirmed that, like all HU-like proteins, mgHU interacts with DNA as a dimer (see the Supporting Information).

At the physiological ionic strength, mgHU binds DNA bulges at least 100-fold stronger than dsDNA. Our experimental approach ensures that it is the major such protein in the *M. gallisepticum* extracts. Other mismatch binders (if any) should have much lower concentrations and/or binding constants. Although the His-tagged purified mgHU and the intact mgHU exhibit identical DNA binding patterns, the cell extracts were generally preferred for a more detailed mgHU characterization because the histidine tag potentially can interfere, for instance, in protein−protein interactions during cooperative binding as well as in DNA binding.

Binding of mgHU to DNA Lesions Is Strong at the Physiological Ionic Strength. It is known from earlier studies that the nonspecific binding of the *E. coli* HU protein to intact dsDNA rapidly weakens with an increased salt concentration and essentially disappears at the "physiological" concentrations of monovalent ions.[7,23,25] In contrast, the specific HU binding to noncanonical dsDNA structures remains significant even at high salt concentrations.[20,21] The salt dependence of binding of mgHU to some representative dsDNA lesions is examined in Figure 5. From the relative intensities of the two marked bands, it can be seen that the binding of mgHU to DNA lesions remains strong and specific at physiological salt concentrations (100−200 mM NaCl). The exact profile depends upon the DNA structure. Figure 5 (bar plots) shows that, with NaCl concentrations increased from 20 mM to 200 nM, mgHU exhibits 4- and 4.5-fold reductions in affinity for bulge A1 and the CC mismatch, respectively. Binding to bulge A7 looks qualitatively different. In this case, the affinity increases with salt concentration and passes through a maximum at 100 mM NaCl. The origin of this effect is unclear.

Binding to DNA Lesions Commonly Targeted by the MMR Pathway. Replication errors lead to DNA lesions of a few definite types. The most frequent are single-base insertions and single-base pair mismatches. Longer similar lesions occur with progressively decreasing probabilities. In *E. coli* and most other bacteria, all these lesions constitute MutS recognition

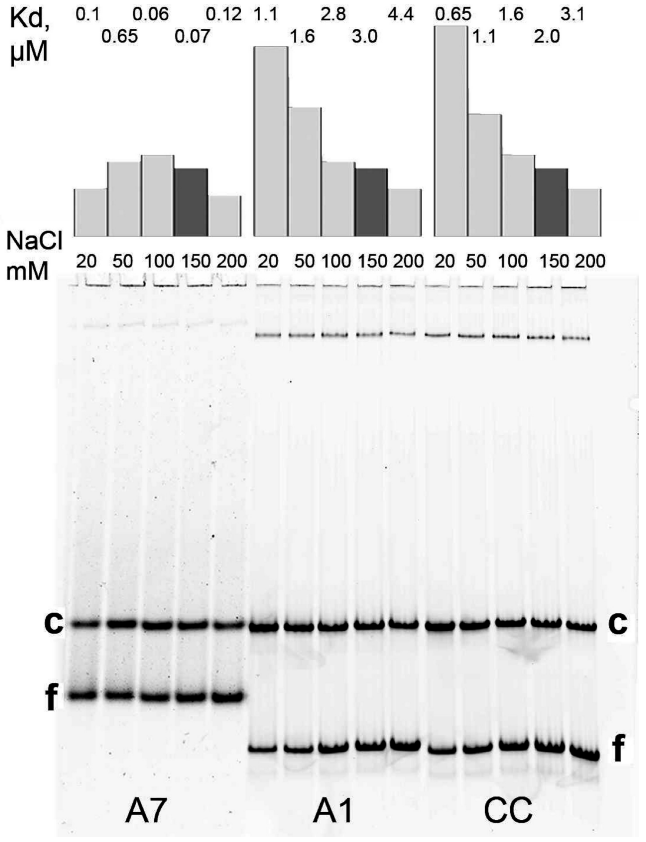

Figure 5. Salt dependence of binding of mgHU to DNA. Three dsDNA substrates indicated at the bottom of the gel were mixed with the *M. gallisepticum* extract (0.1, 2.5, and 2.5 μL per lane for A7, A1, and CC, respectively) in buffers containing different concentrations of NaCl (shown on top of the lanes) and loaded onto a 100 mM Tris-borate buffered gel. The top part of the figure shows the bar plots of the relative intensities of the bands corresponding to mgHU−dsDNA complexes. Each series of bars was normalized separately; that is, they cannot be used for comparing different DNA substrates. The corresponding dissociation constants are shown on the top of the plot in micromolar.

targets and start the MMR pathway (for reviews, see refs 36 and 37). *M. gallisepticum* does not have genes encoding MutS, but the previous experiments show that most of the corresponding DNA lesions are recognized by the mgHU protein. Figure 6 demonstrates that mgHU specifically binds DNA bulges, with an affinity that increases with insert length. The effect is sequence-dependent. For homopolymer bulges of two to seven bases, the mgHU−DNA affinity decreases as follows: T = C > A > G. For the most frequent one-nucleotide bulges, the mgHU binding preference is somewhat different: A = T > C (K_d values of 1, 0.7, and 3 μM, respectively). The G bulge is not recognized (indistinguishable from nonspecific dsDNA binding).

The G bulge exception may have important biological consequences; we therefore checked if the effect was occasional or due to a particular sequence context. Figure 7A compares the binding of mgHU to G bulges of various lengths inserted in two different sites on the same dsDNA fragment. The results are similar, indicating that this effect is local and that mgHU cannot recognize single G bulges. G bulges were similarly inserted in 10 alternative positions within the dsDNA, and in all cases, only weak binding to mgHU was detected (not shown).

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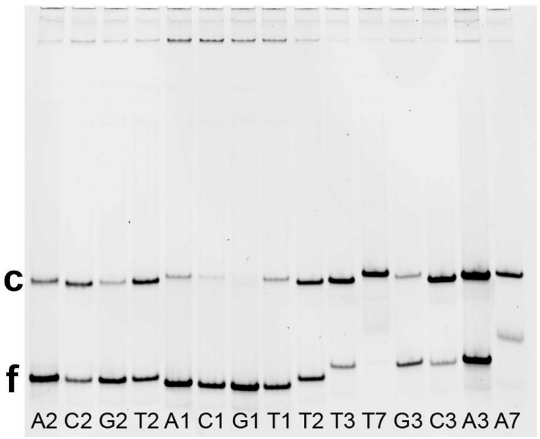

Figure 6. Binding pattern of mgHU–DNA bulges of different lengths and sequences. The *M. gallisepticum* extract (0.25 μL per lane) was incubated in 150 mM NaCl with a series of labeled dsDNA constructs carrying the inserts of one, two, and three indicated bases in the middle. The resulting DNA-containing components were separated via gel. The notation is similar to that in Figure 1.

The binding of mgHU to substitution mismatches of different lengths and sequences was analyzed by band shift assays as demonstrated in Figures 4 and 7B. These data can be assembled in the following series of weaker binding: mismatch 4 = mismatch 3 = mismatch 2 = CC > CT = TT > AA = AC (see Table 1 for K_d values). Binding to mismatches AG, GG, and TG is indistinguishable from nonspecific interactions with intact dsDNA. Like that with bulges, the binding of mgHU to substitution mismatches is independent of sequence context (see Figure 7C).

Table 1. Dissociation Constants of mgHU Complexes with DNA Substitution and Insertion Mismatches (in nanomolar) at 150 mM NaCl

substitution mismatches		bulges (IDLs)		
mismatch of one base		length 1	A	1000
CC	2000		T	700
CT	5000		C	3000
TT	10000		G	ds[a]
AC	30000	length 2	A	1300
AG	ds[a]		T	160
GT	ds[a]		C	150
AA	ds[a]		G	2500
		length 3	A	200
			T	100
mismatch of 2–4 bases			C	35
mismatched bases			G	1000
5′-AC-3′/5′-CC-3′	2500	length 7	A	70
5′-ACG-3′/5′-TCC-3′	3500		T	20
5′-ATGT-3′/5′-GTCC-3′	2000		C	20
			G	500

[a]Weak interaction similar to the nonspecific binding to double-stranded DNA.

The absolute values of the dissociation constants of different mgHU–DNA complexes were evaluated by comparing the relevant band intensities to that of the mgHU–A7 complexes added on the same gel. For the A7 bulge, the dissociation constant was accurately measured as explained above (see Table1 and Materials and Methods).

mgHU dsDNA and ssDNA Binding Site. The size of the mgHU binding site was estimated by measuring its binding to

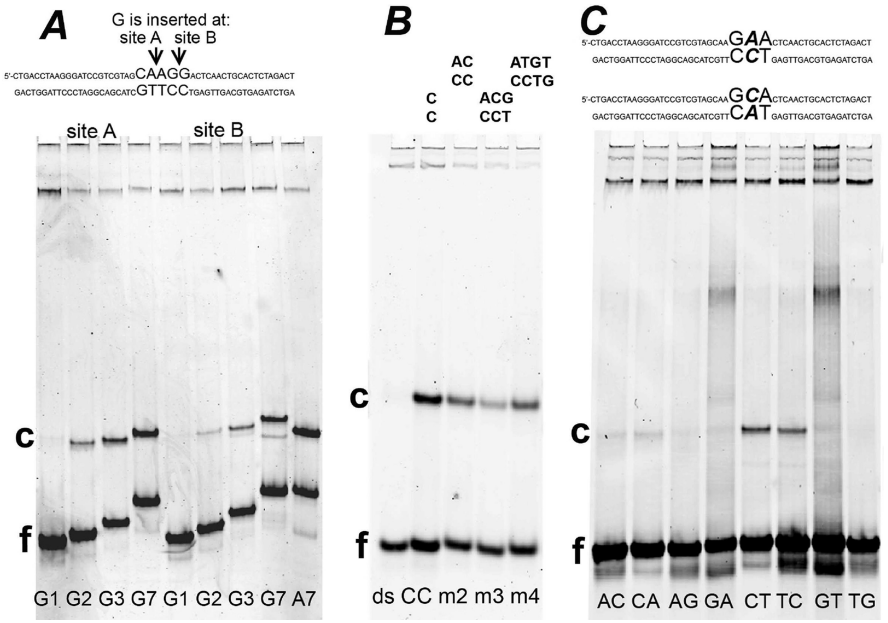

Figure 7. Pattern of binding of mgHU to DNA substitution and insertion mismatches of different lengths, sequences, and contexts. The *M. gallisepticum* extract (0.5, 0.25, and 0.1 μL per lane for panels A–C, respectively) was incubated in 150 NaCl with a series of labeled DNA constructs ,and the resulting DNA-containing components were separated via gel. The dsDNA sequences used are detailed in Materials and Methods. The notation is similar to that of Figure 1. (A) G bulges of various lengths were introduced into two different sequence contexts shown at the top. The insert lengths are indicated below the lanes. Similar results are shown for bulge A7 for comparison. (B) From left to right, lanes ds, CC, m2, m3, and m4 display the band shifts for dsDNA and substitution mismatches of one, two, three, and four bases, respectively; the mismatched bases are indicated at the top. (C) Comparison of binding of mgHU to single-base substitution mismatches placed in two different sequence contexts shown at the top for AC and CA mismatches.

dx.doi.org/10.1021/bi2009097 | *Biochemistry* 2011, 50, 8692–8702

undamaged DNA substrates of various lengths. Binding of HU-like proteins to canonical dsDNA is considered nonspecific because it is weak at physiological and high salt concentrations.[7,23,25] For mgHU, only traces of binding are seen under these conditions (Figures 1 and 4). Therefore, in accordance with the earlier studies, the band shift assay with mgHU−dsDNA and mgHU−ssDNA complexes was conducted at low salt concentrations (40 mM NaCl and 25 mM Tris-borate buffered gel). The results are shown in Figure 8.

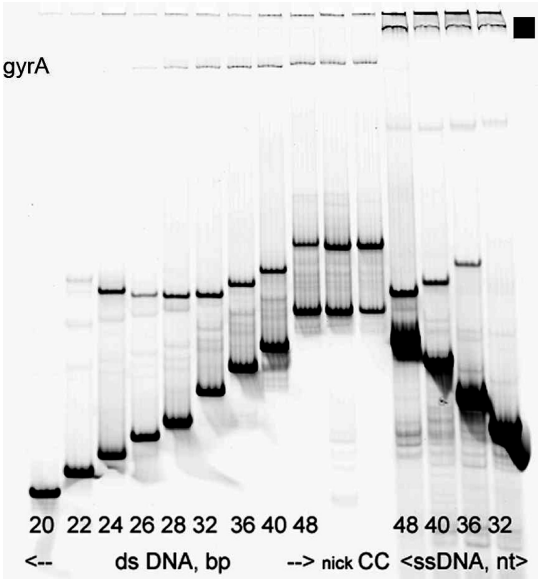

Figure 8. Evaluation of the size of the mgHU binding site for ds- and ssDNA. Labeled DNA substrates of appropriate lengths were incubated with cell-free *M. gallisepticum* extracts (2.5 μL per lane) in 40 mM NaCl, and the resulting compositions of the mixtures were analyzed by polyacrylamide gel electrophoresis (25 mM Tris-borate). The lengths of the DNA fragments are indicated below the lanes. The black rectangle marks the complex with the unidentified ssDNA binding protein.

It is seen that binding to dsDNA is noticeable for lengths of 48, 40, 36, 32, 28, 26, and 24 bp. For 22 bp, it is significantly weaker, and no binding was detected for 20 bp dsDNA. This pattern suggests that the minimal binding site size of mgHU on dsDNA is 22 bp. According to X-ray data,[18,19] 22 bp is exactly the length of a fragment necessary for simultaneous contacts with two binding zones at the opposite sides of the protein. Between 22 and 28 bp, the binding energy should increase rapidly because of the growing number of direct protein−DNA interactions. Beyond 28 bp, the contact surface is complete but the binding can still improve because of multiple binding modes (a single mode for 22 bp DNA and 37 modes for 48 bp DNA). The apparent dissociation constant under low-salt conditions (40 mM NaCl) decreases from 10000 to 5000 nM for dsDNA lengths from 32 to 48 bp, respectively. This picture qualitatively agrees with the data in Figure 8. A similar pattern was previously observed for some HU-like proteins,[38] but not for *E. coli* HU that can bind dsDNA with a binding site of only 9 bp.[18,39−43] *E. coli* HU is characterized by cooperative multimer binding on dsDNA longer than the minimal binding site.[25,44] The last four lanes in Figure 8 demonstrate that mgHU also interacts with ssDNA for chain lengths of ≥36 nucleotides. Binding to 48- and 40-nucleotide fragments occurs with apparent dissociation constants of 8000 and 10000 nM, respectively (in 40 mM NaCl). We conclude that binding of mgHU to ssDNA requires at least 36 nucleotides, which is significantly longer than the 24-nucleotide ssDNA binding site estimated for *E. coli* HU under similar conditions.[26]

Complementation Tests. *E. coli* cells carrying simultaneous deletions of the two genes encoding HU, namely, *hupA* and *hupB*, are characterized by a slow growth phenotype.[45] This phenotype is explained by the recent characterization of the *E. coli* HU regulon that is shown to be composed of four classes of genes responding to anaerobiosis, acid stress, high osmolarity, and SOS induction.[12] The observation that a single deletion, either in *hupA* or in *hupB*, does not impair growth significantly[45] has been used to conduct successful complementation tests with single homologous or heterologous *hup* genes.[17,46]

In this study, the *HimA/Hup_2* gene was selected by the capacity of its product mgHU protein to recognize DNA lesions specific to the MMR pathway. This finding suggests that mgHU is involved in DNA repair. Considering the known pleiotropic function of *E. coli* HU, it is essential to verify that mgHU possesses similar capacities. To this end, we tested if HimA/Hup_2 and HimA/Hup_1 proteins are able to substitute *E. coli* HU. We have expressed individually these two potential *M. gallisepticum* HU-encoding genes, *HimA/Hup_2* and *HimA/Hup_1* from plasmids carrying the arabinose-inducible P_{ARA} promoter, pJO193 and pJO201, respectively (see Materials and Methods). The negative control consisted of parental vector pBADN.[12] As a positive control, we used pBAD-6H-*hupA*, expressing *E. coli hupA* from the same promoter.[46] The four plasmids were transformed into *hupAB* strain JO3020 to yield JO193, JO201, JO215, and JO217. These strains were then plated on complete medium in the presence and absence of arabinose. In the presence of the inducer, we observed that strain JO193, expressing *M. gallisepticum* gene *HimA/Hup_2*, presented a growth phenotype similar to that of the positive control strain JO215 expressing *hupA* (Figure 9); on the other hand, JO201 did not show complementation. In the absence of arabinose, the four strains displayed a typical *hupAB* slow growth phenotype.

Expression of *hup_1* and *hup_2* in *M. gallisepticum*. Most mycoplasmas encode only one HU homologue. Despite the presence of two homologous proteins in *M. gallisepticum*, only one, hup_2, was shown to bind DNA mismatches and to complement *E. coli hupAB*. Quantitative PCR experiments showed that both *hup_1 and hup_2* were expressed, but the content of *hup_2* mRNA was significantly higher than that of *hup_1* mRNA (8-fold). Also, both hup_1 and hup_2 polypeptides were detected in the *M. gallisepticum* proteome.[47] Therefore, hup_1 must play an important role, but it remains unclear.

DISCUSSION

Mycoplasmas are the smallest-known microorganisms characterized, among other features, by the absence of key elements of the MMR system responsible for recognition and correction of replication errors such as base substitutions.[31] Nevertheless, screening of the cell-free extracts of *M. gallisepticum* revealed a protein that recognized single-base mismatches in dsDNA. This factor was identified as a homologue of the HU protein. Similar proteins are ubiquitous in bacteria. The *E. coli* HU has been actively studied over the past four decades.[12,18,19,24,48−52] In recent years, it became clear that HU-like proteins from different species are not identical. On the contrary, the corresponding DNA binding profiles strongly vary among bacteria.[13]

The binding specificity of HU-like proteins can strongly and sometimes unpredictably change because of even single-amino acid substitutions.[38−40,53,54] Sequence comparison reveals that mgHU has multiple modifications in conserved regions considered critical for the protein structure and specificity (see Figure 3). Three

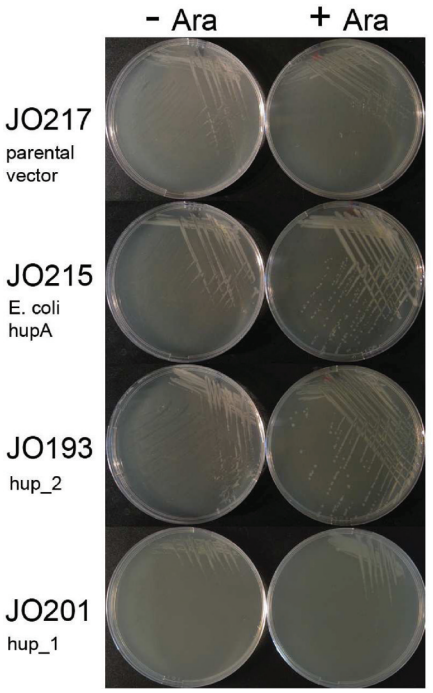

Figure 9. Complementation test. *E.coli hupAB* strain JO3020 was transformed with plasmids expressing *M. gallisepticum* HU-encoding genes, *hup_2* (JO193) and *hup_1* (JO201), *E. coli hupA* (JO215) under arabinose-inducible P_{ARA} promoter, and the parental vector (JO217). In the absence of arabinose, the four strains displayed the typical *hupAB* slow growth phenotype (left). In the presence of 0.2% arabinose (right), strain JO193, expressing *M. gallisepticum* gene *hup_2*, presented a growth phenotype similar to that of the positive control strain JO215 expressing *E. coli hupA*, while strain JO201 expressing *M. gallisepticum* gene *hup_1* displays the slow growth phenotype.

features deserve particular attention. (i) The conserved motif GFGnF (positions 46−50) and invariant F79 form the hydrophobic core of the HU dimer.[18] The sequence alignment shown in Figure 3 suggests that the hydrophobic core of the mgHU dimer significantly differs from that in the available X-ray structures. (ii) The N-terminal sequence of mgHU is very dissimilar to that in all earlier studied HU-like proteins. The highly conserved K3 commonly plays a key role in stabilizing the bent DNA conformation.[38] The computer alignment suggests that in mgHU it is absent and a MAKIKS hexapeptide extension is added to the N-terminus. The level of sequence homology is fairly low, however, and one cannot exclude the possibility that K3 of mgHU in fact corresponds to the conserved position; however, there is an extra hexapeptide loop elsewhere near the N-terminus. (iii) The mgHU sequence has a functionally important R61I substitution in the GRNPnT motif (positions 60−65). According to X-ray data, the intercalating P63 plays a key role by providing a kink in the bound DNA molecule.[18] The R61V substitution was earlier studied for *Thermotoga maritima* HU.[53] It appeared that a nonpolar residue at this position reduces the affinity of HU for intact dsDNA. These three features are recurrent in the HU homologues of mollicutes.

In spite of significant sequence modifications, mgHU shares the main properties of HU-like proteins and is consistent with the previously studied HU homologues from different species. This protein avidly binds nicks, ssDNA, and various non-canonical structures such as four-way junctions and ds- and ssDNA forks (D. Kamashev, unpublished results). Binding to all these substrates is strong over a wide range of salt concentrations, including the physiological concentration. Although mgHU binds intact dsDNA poorly, with a qualitative difference from the *E. coli* HU with regard to the cooperativity and size of the binding site, this is evidently a histone-like protein because it can substitute for the *E. coli* HU in vivo.

In contrast, the second annotated HU homologue in mycoplasma genomes (HimA/Hup_1) apparently has another function. Regardless of salt concentration, it does not bind DNA substrates used in this study and cannot substitute for *E. coli* HU in complementation experiments. The very presence of two HU homologues in mycoplasmas is surprising. A single such gene is sufficient for most bacteria, whereas the *Mycoplasma* genus emerged via reductive evolution accompanied by the loss of numerous genes unnecessary for the specific mode of life of these organisms.[27] However, other bacteria commonly employ a group of nucleoid-associated proteins, including HU, IHF, Fis, H-NS, and Lrp. These proteins harbor distinct and often complementary activities. For instance, H-NS is known to suppress genes by causing DNA to loop near promoter regions, while the *E. coli* HU is able to straighten DNA and perhaps serves as an H-NS antagonist.[55] Because none of these proteins, except HU, is present in mycoplasma genomes, it is possible that the HimA/Hup_1 gene product has evolved to substitute for some of the missing architectural activities.

Additional studies are necessary to clarify the structural origin of the altered substrate specificity of mgHU. The sequence dependence of mismatch binding correlates with the relative flexibility of typical MMR substrates.[36] It is possible that dsDNA flexibility is the governing factor in the specific recognition of small lesions by mgHU. However, kinked and prebent substrates are preferred by most HU-like proteins, and it is difficult to explain why the same small difference in flexibility is much more important for mgHU. It is also possible that the recognition of base pair mismatches occurs when the invariant P63 intercalates into the base pair stack. The altered specificity can be provided by proximal amino acids as in the case of the R61V substitution studied previously in *T. maritima* HU.[53] The local interactions in the intercalation site can explain the relatively weak binding of mgHU to all mismatches and IDLs, including guanine.

The structure specific interactions of HU-like proteins with noncanonical dsDNA motifs were first discovered by screening bacterial extracts for binding activities toward cruciforms and gaps.[20,21] The group of specifically recognized substrates also involves dsDNA forks, 3′-overhangs, free ssDNA, and long stretches of mispaired bases within dsDNA.[23,26] The functional role of these interactions remains elusive. The motifs recognized always carry a kink or a hinge point; therefore, the strong specific binding could be a side effect of the nonspecific bending ability necessary for histone-like functions of HU proteins.[56,57] It was noticed, however, that essentially all specifically recognized structures represent intermediates of DNA repair pathways, suggesting that HU recognition of distorted dsDNA is somehow involved in these processes.[9,21,23] The participation of HU proteins in recombination repair has been confirmed,[58] and this activity is probably independent of the histone-like functions.[10]

The increased flexibility is characteristic of extended substitution mismatches and bulges. These motifs are often used for constructing DNA fragments with increased affinity for HU.[18,26,53] Because such substrates do not exist in the living cell, the binding could be considered as a predictable in vitro effect. Biologically important single-base mismatches increase DNA flexibility only slightly. In most organisms, these lesions are specifically recognized by the MutS protein of the MMR system. Interestingly, the MutS binding

affinity decreases with DNA flexibility, and the most flexible single CC mismatches are the worst MMR substrates.[36] This observation prompted specific screening for CC mismatch binding activities in *E. coli*.[59] It appeared that CC mismatches could be recognized by FabA and MutM proteins and not by HU. In vitro HU binding was checked for the single TT mismatch[60] and the double TG·CT mismatch.[26] In both cases, the binding appeared to be similar to that for nonspecific dsDNA.

Among the known mismatch binders, only MutM has an annotated homologue in the reduced genome of *M. gallisepticum*. Because MutM was not detected in our screening assays, its activity is negligible because of either a low concentration or an altered specificity. The latter would not be surprising given the example revealed here of the *HimA/Hup_1* gene product. At the same time, the properties of the second HU homologue (mgHU) have evolved so that it can distinguish single-substitution and insertion mismatches in the presence of a large excess of dsDNA. mgHU is abundant in the cell because it is the only NAP available; therefore, the DNA lesions resulting from typical replication errors should be recognized very efficiently. We believe that the acquired specificity toward these DNA lesions is related to the absence of the MMR system in mycoplasmas. For instance, the bacterium *Acholeplasma laidlawii* belongs to the same Mollicutes class as *M. gallisepticum*, but it has the MMR system. Preliminary data indicate that the *A. laidlawii* HU homologue does not have a specific preference for MMR substrates (ref 61 and unpublished results of D. Kamashev). It is possible that this property of mgHU is somehow used for reducing mutation rates because the mutation rates are probably similar in mycoplasmas and *E. coli*.[33,34]

■ ASSOCIATED CONTENT

Supporting Information

Supplementary experimental methods and results mentioned in the text, including one table and five figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by State Contracts 14.740.11.0763 and 16.740.11.1371.

■ ACKNOWLEDGMENTS

We thank Dr. Josette Rouviere-Yaniv for kindly supplying us with *E. coli* HU protein.

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