Functional Identification of Toxin-Antitoxin Molecules from *Helicobacter pylori* 26695 and Structural Elucidation of the Molecular Interactions

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Bacterial toxin-antitoxin (TA) systems are associated with many important cellular processes including antibiotic resistance and microorganism virulence. Here, we identify and structurally characterize TA molecules from the gastric pathogen, *Helicobacter pylori*. The HP0894 protein had been previously suggested, through our structural genomics approach, to be a putative toxin molecule. In this study, the intrinsic RNase activity and the bacterial cell growth-arresting activity of HP0894 were established. The RNA-binding surface was identified at three residue clusters: (Lys8 and Ser9), (Lys50–Lys54), and (Arg86 and His84–Phe88). In particular, the -UA- and -CA- sequences in RNA were preferentially cleaved by HP0894, and residues Lys52, Trp53, and Ser85–Lys87 were observed to be the main contributors to sequence recognition. The action of HP0894 could be inhibited by the HP0895 protein, and the HP0894–HP0895 complex formed an oligomer with a binding stoichiometry of 1:1. The N and C termini of HP0894 constituted the binding sites to HP0895. In contrast, the unstructured C-terminal region of HP0895 was responsible for binding to HP0894 and underwent a conformational change in the process. Finally, DNA binding activity was observed only for HP0895 and the HP0894–0895 complex but not for HP0894 alone. Taken together, it is concluded that the HP0894–HP0895 protein couple is a TA system in *H. pylori*, where HP0894 is a toxin with an RNase function, whereas HP0895 is an antitoxin functioning by binding to both the toxin and DNA.

*Helicobacter pylori* has a unique ability to survive in an acidic environment and to colonize the gastric mucosa (1). It can cause diverse gastric diseases such as peptic ulcers, chronic gastritis, mucosa-associated lymphoid tissue lymphoma, and gastric cancer (2–4). Approximately half of the world population is infected by *H. pylori*; therefore, it is an important human bacterial pathogen. Our previous study (5) reported on the solution structure of the conserved hypothetical protein HP0894 from *H. pylori* 26695 as part of the structural genomics efforts of our group. In that study, we stated that HP0894 shares high structural similarity with archaeal RelE (aRelE), the toxin of the chromosomal toxin-antitoxin (TA) system *Pyrococcus horikoshii*, and low but marked structural similarity with guanyloribonuclease of *Streptomyces aureofaciens* (RNase Sa). Those findings prompted us to speculate that HP0894 might act as a TA toxin with intrinsic ribonuclease (RNase) activity.

Many TA modules have been identified on the chromosomes of archaea and bacteria, as well as on plasmids (6, 7). These TA modules consist of a stable toxin and an unstable antitoxin. In a typical TA system, toxin expression induces arrest of cell growth, whereas the antitoxin neutralizes the toxin by a direct protein-protein interaction (6). Generally, both proteins are encoded within a single operon, with the toxin gene usually located directly downstream of the antitoxin gene (8). In most cases, TA operons are negatively autoregulated at transcription level by antitoxins and toxin-antitoxin complexes, which bind to the TA locus promoters (6). TA systems on plasmids are involved in plasmid stabilization through toxin-mediated post-segregation killing of plasmid-free progeny cells (9). The role of chromosomal TA systems is less clear: however, the most accepted hypothesis is that chromosomal TA systems act as stress regulators, active under nutritional and/or environmental stresses (6, 9, 10). Thus, TA systems in bacterial pathogens may be a good target for antibiotics (11).

The *Escherichia coli* K12 chromosome possesses at least five confirmed and relatively well characterized TA pairs.
(RelE-RelB, YafQ-DinJ, YoeB-YefM, MazF-MazE, and ChpBK-ChpBI) (6). The RelE, YafQ, and YoeB toxins are classified within the same family (RelE family) due to low but significant sequence similarities among them (6). Likewise, the MazF and ChpBK toxins share sequence similarity and are classified in another family (MazF family) (6). These toxins function, or are supposed to function, by inhibiting translation through mRNA cleavage (6). Among those toxins, YoeB, YafQ, MazF, and ChpBK have intrinsic RNase activity (12–16), whereas RelE does not. RelE is a ribosome-dependent endoribonuclease that is active when it associates with a ribosome (17, 18) or a stimulatory factor for the intrinsic endoribonuclease activity of a ribosome (12, 19). YoeB possesses purine-specific endoribonuclease activity in vitro, but according to a recent study, it binds to the 50 S ribosomal subunit in 70 S ribosomes and interacts with the ribosomal A site, leading to mRNA cleavage at that site (20). YafQ was also shown to associate with ribosomes and to cleave mRNA in a sequence-specific and frame-dependent manner (13). In contrast, MazF and ChpBK function by cleaving mRNAs independently of the ribosome, in a sequence-specific manner (15, 16). The crystal structures of E. coli YoeB, YoeB-YefM, MazF-MazE, and the P. horikoshii aRelE-aRelB complex (the archaeal homologues of E. coli RelE-RelB), as well as the nuclear magnetic resonance (NMR) structures of E. coli RelER83A/R85A and E. coli RelER83A/R85A-RelB C-terminal peptide (RelE-RelBc) complex, have been solved (7, 12, 21, 22). The aRelE, RelE, and YoeB toxins share high structural similarity among them; however, the structures of YafQ or its homologues have not yet been determined for any archaea or bacteria.

This study investigates the speculation that HP0894 acts as a TA toxin with intrinsic RNase activity. Herein, we identify the biological function of HP0894 and characterize several structural aspects of its catalytic and inhibitory mechanisms.

**EXPERIMENTAL PROCEDURES**

**Preparation of Protein Samples**—Subcloning and overexpression were carried out as described previously for HP0894 (5). For the production of 15N-, 15N, 13C-, or 15N, 2H (70%)-labeled proteins, M9 medium containing 15NH4Cl, [13C]glucose, and/or D2O as stable isotope sources was used. The protein samples were purified using a Ni2+-affinity column following standard protocol. The same procedures were used to prepare HP0894 and mutated HP0894. All resulting HP0894 and HP0895 constructs contained 8 non-native residues (LEHHHHHH) at the C terminus (referred to as HP0894-His and HP0895-His, respectively). Those residues facilitated subsequent protein purification.

**Construction of HP0894-HP0895 and HP0894-HP0316 Co-expression Systems**—The full-length hp0895 and hp0894 genes were amplified from H. pylori genomic DNA by PCR as different DNA fragments and were subsequently subcloned under the T7 promoter in pET21a (ampicillin-resistant) and pET29a (kanamycin-resistant) vectors (Novagen, Madison, WI), respectively. These two vectors were co-transformed into E. coli BL21 (DE3) (HP0894-HP0895 co-expression system). Using the same procedure, co-expression systems composed of HP0894 (in vectors pET15b (Novagen) or pET21a) and HP0316 (in pET29a) were constructed (HP0894-HP0316 co-expression system). In all cases, the HP0894 construct contained 8 non-native residues including a His tag at the C terminus (referred to as HP0894-His) or 20 non-native residues including a His tag at N terminus (referred to as His-HP0894). The HP0895 and HP0316 construct contained no additional residues.

**Co-expression and Co-purification of HP0894-HP0895 or HP0894-HP0316**—HP0894-HP0895 and HP0894-HP0316 were co-expressed and co-purified using the co-expression systems described above. Bacteria were grown at 37 °C in LB broth containing antibiotics (50 μg/ml ampicillin and 40 μg/ml kanamycin). Induction, cell harvest, cell lysis, and purification with fast protein liquid chromatography (FPLC) Ni2+-affinity columns were conducted following standard protocols. For further purification, gel filtration chromatography was performed. The eluted fractions from the Ni2+-affinity gel and gel filtration columns were analyzed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Toxicity of HP0894 on E. coli**—E. coli BL21 (DE3) cells harboring pET21a plasmids without subcloned genes (pET21a), the HP0894 expression plasmids (HP0894), and the HP0894-HP0895 co-expression plasmids (HP0894+95) were grown at 37 °C in 100 ml of LB broth containing antibiotics (50 μg/ml ampicillin for pET21a and HP0894 and 50 μg/ml ampicillin and 40 μg/ml kanamycin for HP0894+95). At an A600 of about 0.4, 0.5 μl isopropyl-β-D-thiogalactopyranoside was added. Subsequently, samples for viable counts and SDS-PAGE analyses were taken at several time points. Viable counts were made by plating dilutions of the cultures onto LB plates containing appropriate antibiotics. In addition, the cells harvested from 125 μl of the samples taken at each time point underwent SDS-PAGE.

**In Vitro RNA Cleavage by HP0894**—A DNA fragment containing the T7 promoter and the hp0893 gene was obtained by PCR amplification from H. pylori genomic DNA. The hp0893 mRNA was synthesized in vitro from this DNA fragment using the T7-MEGAscript kit (Ambion, Austin, TX). Mixtures of hp0893 mRNA (~310 bases, 300 ng) HP0894, HP0894 mutants, HP0895, and/or bovine pancreatic ribonuclease A were prepared in several ratios in 20 mM Tris-HCl (pH 7.4) buffer containing 150 mM NaCl (total volume, 5 μl). The reaction was performed at 37 °C for 60 min and was stopped by adding 5 μl of the sequencing loading buffer (Am- bion). If necessary for denaturing the proteins, the sample was further mixed with 0.1 μl of 2-mercaptoethanol and 0.9 μl of SDS solution (4.4%, w/v). The samples were loaded and electrophoresed on a 1.5% agarose gel containing ethidium bromide with Tris borate/EDTA buffer.

**DNA Binding Test**—A 150-bp DNA fragment located upstream of hp0895 and including the hp0895-hp0894 promoter region was amplified by PCR from H. pylori genomic DNA. HP0894, HP0895, and the DNA fragment were mixed in several ratios in 20 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl (total volume, 10 μl) and incubated for 15 min at room temperature. Following incubation, 2 μl of loading buffer were added to each mixture, and electrophoresis was
HP0894-HP0895 Is a Toxin-Antitoxin System

performed with 2% native agarose gel containing ethidium bromide in Tris borate/EDTA buffer.

**Gel Filtration Chromatography**—HP0894, HP0895, the mixture of HP0894 with HP0895, and fully purified HP0895-HP0894 complex were separately fractionated on a Biosep SEC S-3000 or S-2000 column (Phenomenex, Torrance, CA) using a Hitachi L 6200 HPLC system. Mobile phases comprised 20 mM sodium phosphate or acetate buffers with various pH levels and concentrations of NaCl. The proteins were eluted at flow rates of 0.4 or 0.8 ml/min and were monitored using UV at 220 or 280 nm.

**NMR Spectroscopy and Titrations**—For HP0894 titration against HP0894, two-dimensional $^{[1}H-^{15}N$ heteronuclear single quantum coherence (HSQC) spectra of $^{15}N$-labeled HP0894 alone (final concentration: 0.2 mM), and with HP0895 added incrementally (0.13, 0.16, 0.25, and 0.5 mM) to the sample were obtained on a Bruker Avance 500 spectrometer (Bruker AXS, Madison, WI). In addition, two-dimensional $^{[1}H-^{15}N$ transverse relaxation optimized spectroscopy (TROSY) spectra of $^{15}N$, $^{2}H$ (70%)-labeled HP0894 alone (final concentration: 0.2 mM) and with HP0895 added incrementally (0.04, 0.08, 0.12, 0.16, and 0.25 mM) to the sample were obtained on a Varian VNMRS 900 spectrometer (Varian, Palo Alto, CA).

For HP0895Ctp titration against HP0894, a 30-residue peptide corresponding to the C-terminal region (Val96–Ser125) of HP0894 was commercially synthesized and purified (96% purity). Two-dimensional $^{[1}H-^{15}N$ TROSY spectra of $^{15}N$-labeled HP0894 alone (final concentration: 0.1 mM) and with HP0895Ctp added incrementally (0.05, 0.1, and 0.2 mM) to the sample were obtained on a Bruker Avance 600 spectrometer.

For single-stranded DNA (ssDNA) titration against HP0894, 10-base ssDNA oligomers with various base sequences were synthesized. Two-dimensional $^{[1}H-^{15}N$ TROSY spectra of $^{15}N$-labeled HP0894 alone (final concentration: 0.1 mM) and with the ssDNA oligomers added incrementally (up to 0.4 mM) were obtained on a Bruker Avance 500 spectrometer. The averaged chemical shift changes were calculated by the following equation from Ref. 23.

$$\Delta \delta_{av} = [(\Delta \delta_{HH})^2 + (\Delta \delta_{HN}/6.57)^2]^{0.5}$$  \hspace{1cm} (Eq. 1)

For backbone resonance assignments of HP0894 bound with HP0895Ctp, three-dimensional TROSY-type HNCA (trHNCA), three-dimensional $^{15}N$-separated nuclear Overhauser enhancement spectroscopy (NOEYS)-HSQC, and HNHA spectra of $^{13}C$, $^{15}N$- or $^{15}N$-labeled HP0894 (0.2 mM) mixed with HP0895Ctp (0.3 mM) were obtained on a Varian VNMRS 900 (for three-dimensional trHNCA) or a Bruker Avance 600 spectrometer (for NOEYS-HSQC and HNHA).

All NMR samples were dissolved in 20 mM sodium phosphate/pH 5.0 buffer containing 150 mM NaCl except for HP0895 titration (pH 5.0, 500 mM NaCl). These NMR samples contained 10% D$_2$O for the lock signal. All spectra were processed and analyzed using NMRPipe/NMRDraw (24) and NMRView (25), respectively.

**Circular Dichroism**—The circular dichroism (CD) analyses was performed on the purified proteins and their mixtures using a JASCO J-715 spectropolarimeter (JASCO, Easton, MD). Standard far-UV or near-UV CD spectra of the proteins in 20 mM sodium phosphate buffers were recorded (20 °C, scan speed 50 nm/min, 0.5-nm step resolution) at various pH levels and NaCl concentrations using 5- or 1-mM-long cells. For each CD analysis, the results of three individual scans were averaged.

**Primer Extension**—The hp0893 mRNA was used as a primer template. Two different DNA primers were synthesized and 5’-labeled with $[^\gamma-^{32}P]$ATP using T4 polynucleotide kinase. Primers (0.8 pmol) were annealed to each template (1 pmol) in 10-μl mixture samples by incubation at 90 °C for 5 min followed by slow cooling to room temperature. The reaction mixture was digested by adding 1 μl of various amounts of HP0894 at 37 °C for 1 h. Subsequent primer extension reactions were performed by adding 4 μl of 5× buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, and 15 mM MgCl$_2$), 5 mM DTT, 0.5 mM dNTP, and 20 units of SuperScript III reverse transcriptase (Invitrogen) for a final reaction volume of 20 μl. The cDNA was synthesized at 55 °C for 30 min and then purified by phenol/chloroform extraction and ethanol precipitation. The products were then subjected to 8% denaturing PAGE and analyzed by autoradiography.

**RESULTS**

**HP0894 and HP0895 Proteins Form a Stable Complex**—On the H. pylori chromosome, the hp0895 gene is located directly upstream of the hp0894 gene (Fig. 1A). We expected that if HP0894 is a TA toxin, then it would bind to HP0895, a hypothetical protein and putative TA antitoxin of H. pylori. To identify the potential interaction between HP0894 and HP0895, both proteins were co-expressed from two co-transformed plasmids (HP0894 in pET21a and HP0895 in pET28a) in E. coli BL21 (DE3) and then co-purified. For detection and purification, HP0894 was expressed as a C-terminal His tag fusion (HP0894-His) and HP0895 expressed without a purification tag. SDS-PAGE analysis under a range of imidazole treatments revealed the presence of both expressed proteins (HP0894-His and HP0895) in the same eluted fractions from the Ni$^{2+}$-affinity and subsequent gel filtration columns (Fig. 1B). Thus, the interaction between HP0894 and HP0895 appeared to be stable.

**Inhibitory Effect of HP0894 on E. coli Cell Growth Is Neutralized by HP0895**—The potentials of HP0894 as a toxin and of HP0895 as an antitoxin were examined by measuring their effects on the viability of E. coli cells grown in the presence of isopropyl-β-d-thiogalactopyranoside. As shown in Fig. 1C, the number of viable E. coli cells decreased significantly with expression of HP0894 only, but co-expression of HP0894 and HP0895 neutralized the detrimental effect of HP0894 on E. coli cell growth.

**In Vitro RNase Activity of HP0894 Is Inhibited by HP0895**—To determine whether HP0894 has intrinsic RNase activity, we tested in vitro RNase activity of HP0894 and the inhibitory effect of HP0895. As shown in Fig. 1D, in vitro synthesized mRNA was digested into smaller fragments following incubation with HP0894, whereas the addition of HP0895 inhibited mRNA decay by HP0894. HP0895 alone did not digest mRNA.
and did not affect the activity of RNase A. In addition, the RNase activity of a single mutant HP0894 (H84A) was markedly reduced, indicating that RNA cleavage by HP0894 was not due to contamination of RNases during purification and that the His84 residue is a key catalytic residue of HP0894.

**HP0895 and the HP0894-HP0895 Complex Bind to DNA**—As shown in Fig. 1E, HP0895 alone and the HP0894-HP0895 complex were able to bind to the DNA fragment harboring the appropriate promoter region, and an upward shift was observed. HP0894 alone was not able to bind to DNA.

**Binding Stoichiometry of HP0894 and HP0895**—For identification of the binding stoichiometry of HP0894 and HP0895, two-dimensional [1H-15N] HSQC-based NMR titration of 15N-labeled HP0894 with unlabeled HP0895 was performed on 500-MHz NMR. As shown in [supplemental Fig. S1](#), the HSQC spectra at the 0.65 and 0.83 molar ratios of [HP0895]/[HP0894], the intensities of all of the resonances of 15N-labeled HP0894 were noticeably decreased, but the overall chemical shifts were nearly unchanged when compared with the spectrum of HP0894 alone. At the 1.25 molar ratio, most of the resonances disappeared, and there were few spectral changes between the 1.25 and 2.5 molar ratio results. [Supplemental Fig. S1F](#) shows the intensity changes in the Gly21 and Gln43 resonances, which showed little chemical shift change with the addition of HP0895. The overall aspect of the spectral changes demonstrates that the stoichiometry of interaction between HP0894 and HP0895 is 1:1.

Subsequently, near-UV CD titration was performed. As shown in [supplemental Fig. S2](#), HP0894 (100 μM) shows a characteristic positive peak in the wavelength region of 290–295 nm of its near-UV CD spectrum. Titrations of HP0894 with HP0895 up to 1:1 molar ratio reveal a decrease in the height of that peak. Analysis of changes of the CD intensities of those peaks against the concentrations of added HP0895 yields a 1:1 binding stoichiometry with a dissociation constant (Kd) of ~10⁻⁷ M.
HP0894-HP0895 Is a Toxin-Antitoxin System

Oligomerization States of HP0894, HP0895, and the HP0894-HP0895 Complex—To investigate the oligomerization states of HP0894, HP0895, and the HP0894-HP0895 complex, gel filtration chromatography was performed on HP0894-His (11.4 kDa), HP0895-His (15.8 kDa), and a mixture of the two proteins (HP0894-His was used in excess to ensure complete binding) using 20 mM sodium phosphate buffer (pH 5) containing 500 mM NaCl as a mobile phase. The results indicate that HP0894 exists as a monomer in solution but that HP0895 and the HP0894-HP0895 complex exist as large multimers (supplemental Fig. S3). Based on the elution times of the molecular markers around those of HP0895 and HP0894-HP0895 complex, the molecular masses of them could be roughly estimated as approximate hexamers ((HP0895)_6) and HP0894-HP0895 (HP0894-HP0895)_6.

Subsequently, the effects of pH and salt concentration on the oligomerization states of HP0895 and the HP0894-HP0895 complex were investigated with various mobile phases. As shown in supplemental Fig. S4, under most of the indicated conditions, HP0895 and the HP0894-HP0895 complex still formed large multimers. However, in absence of additional salt, the retention times of HP0895 and the HP0894-HP0895 complex increased markedly, indicating that HP0895 and the HP0894-HP0895 complex were likely to exist in monomeric form. However, the two-dimensional [1H-15N] HSQC spectrum of 15N-labeled HP0894 (50 μM) mixed with unlabeled HP0895 (more than 50 μM) at pH 5 and in the absence of additional salt showed that almost all of the HP0894 resonance peaks disappeared (data not shown). This result indicates that the HP0894-HP0895 complex seems to have a multimeric or aggregated form at NMR scale concentrations, even in the absence of additional salt.

Binding Sites of HP0894 and HP0895—As HP0894 exists as a multimer or as an aggregated form with a high molecular mass in solution, we were not able to get well-dispersed NMR spectra of 15N-labeled HP0895. Therefore, we indirectly identified the region within HP0895 that is responsible for the HP0894-HP0895 interaction. HP0316 (130-residue protein) is homologous to HP0895. The two proteins have an 85% sequence identity with almost identical amino acid sequences except for 35 residues (in HP0316) and 30 residues (in HP0895) in their C-terminal regions (Fig. 2A). We monitored binding between HP0894 with His tag at the N or C termini and HP0316 without any fusion tag by co-expression and co-purification using a Ni2+-affinity column. SDS-PAGE analysis of the elution fraction from the Ni2+-affinity column containing co-expressed HP0316 and HP0894 (amounts of both expressed proteins were nearly the same) did not show a band corresponding to the HP0316, unlike the result with the HP0894-His/HP0895 mixture (Fig. 2B). These results indicate that HP0316 does not bind to HP0894; thus, the 30-residue C-terminal region of HP0895 is responsible for binding with HP0894.

Based on these results, and to confirm the binding of this C-terminal region of HP0895 with HP0894, we synthesized a 30-residue peptide, HP0895Ctp (Val196–Ser125), corresponding to the C-terminal region of HP0895 and investigated the interaction between this peptide and HP0894. A series of two-dimensional [1H-15N] TROSY spectra of 15N-labeled HP0894 was recorded with successive additions of unlabeled HP0895Ctp (0, 0.5, 1, and 2 molar equivalents). As shown in Fig. 2C, obvious chemical shift changes in the slow exchange mode on the NMR time scale were observed for a lot of the residues of HP0894, and some other residues showed chemical shift changes in fast exchange mode. Chemical shift changes from the residues in the slow exchange mode were completed with equimolar HP0895Ctp, whereas fast exchange mode chemical shift changes continued above 1:1 molar ratio in an HP0895Ctp concentration-dependent manner. These results indicate that the dissociation constants (K_d) of HP0895Ctp (and consequently HP0895) with HP0894 are relatively small (~10^-8 M or smaller) (27). Thus, HP0894-HP0895 binding is strong. In addition, these results confirmed that HP0895 binds to HP0894 via its C-terminal region with a binding stoichiometry of 1:1.

A plot of the chemical shift changes against the residues of HP0894 is represented in Fig. 3A. For this plot, the backbone N and H_N resonances of uncomplexed HP0894 were assigned from the data obtained in an earlier study (5). Regarding backbone resonance assignments for HP0895Ctp-bound HP0894, sequential Cα connections determined from three-dimensional trHNCA and H_N(i) – H_N(i+1) and H_N(i) – H_N(i+1) sequential nuclear Overhauser enhancements (NOEs) obtained from three-dimensional 15N-separated NOESY-HSQC and HNHA spectra of 15N- or 15N, 13C-labeled HP0894 in complex with HP0895Ctp were used. The N-terminal residues Leu2, Leu3–Asn20, Phe22–Glu38, and Leu40 and C-terminal residues Val65, Lys72, Leu76, and Leu78–Leu86 of HP0894 show obvious chemical shift changes in slow exchange mode. Overall, the chemical shift changes of the N-terminal residues were more substantial than those of the C-terminal residues (Fig. 3A). These two discrete residue groups in the HP0894 sequence could be mapped structurally into a single consecutive region on a three-dimensional HP0894 NMR structure, which is reasonably suggested to be the HP0895-binding site (Fig. 3B). In addition, rather obvious perturbations in the fast exchange mode were observed for various residues including His84, Ser85, Asp46, and Glu58.

Furthermore, we measured two-dimensional [1H-15N]-TROSY spectra of 15N, 2H (70%)-labeled HP0894 alone and when mixed with unlabeled full HP0894 on 900-MHz NMR. In the presence of a molar excess of HP0895, most backbone amide resonances of labeled HP0894 disappeared. However, as shown in supplemental Fig. S5, within the 0 – 0.8 molar ratios of [HP0895]/[HP0894], detectable fast exchange mode chemical shift changes could be observed in the cross peaks corresponding to His84, Ser85, Asp46, Leu86, and Glu58 residues of HP0894. Slow exchange mode spectral changes were not observed because of the high molecular mass of the complex. These results correspond to the fast exchange mode chemical shift perturbations shown in the titration with HP0895Ctp. In particular, the chemical shift changes in the His84 and Ser85 cross peaks were more marked than those in other residues. The results suggest that these residues undergo conformational changes upon HP0895 binding.
Structural Characterization of HP0894-HP0895 Interaction by CD Spectroscopy—Far-UV CD spectra analyses revealed that HP0895 has a folded structure at various pH values and salt (NaCl) concentrations, and its conformation is stable under the conditions tested (supplemental Fig. S6, A and B). In an analysis based on the CD spectrum at pH 5 without additional salt using the K2d program (29), HP0895 was estimated to be composed of 41% α helix, 20% β sheet, and 39% random coil. However, another CD analysis (supplemental Fig. S6 C) indicated that the 30-residue HP0895Ctp is unstructured. In addition, CD analyses showed that new structure elements were created in the HP0894-HP0895Ctp complex. As the HP0895Ctp-binding region of HP0894 has a well formed secondary structure, it is reasonable to suggest that these created structure elements were from HP0895Ctp. Thus, the unstructured HP0895 C-terminal region transforms into a structured form upon binding to HP0894. Furthermore, far-UV CD titrations of HP0894 with HP0895ctp yield a 1:1 stoichiometry with $K_d$ of $\sim 10^{-8}$ M (supplemental Fig. S6D).

Primer Extension—To identify the sequence of the cleavage site on mRNA by HP0894, HP0894-mediated cleavage of hp0893 mRNA was analyzed by primer extension experiments. As shown in Fig. 4, cleavage occurred predominantly before adenine (A) or guanine (G) residues. Based on the frequency of occurrence, A is preferred to G, and preferentially, the immediate upstream base of the A or G cleavage sites is U or C. Considering the overall tendencies in the sequence around the cleavage site, -U:A- and -C:A- sequences are the most preferred cleavage sites. In addition, when the sites of cleavage related to codons are analyzed, many major cleavage bands that correspond to termination codons (U:AA or U:AG) were observed.

Interaction of HP0894 with RNA Substrate Homologues—To assess the interaction of HP0894 with RNA substrates,
NMR titration experiments were carried out using ssDNA oligomers as RNA substrate homologues because RNA nucleotide oligomers could be cleaved by HP0894 and thus were not suitable for NMR titration study. Chemical shift perturbations of $^{15}$N-labeled HP0894 in two-dimensional $[^{1}H-^{15}N]$-TROSY spectra were monitored by adding a synthetic 10-base ssDNA oligomer (ssDNA-U; d(ACACUAAGAA)) at 0–4 molar ratios of [oligonucleotide]/[HP0894]. In the previous primer extension experiment, cleavage band 12 in Fig. 4A was the most strong, and thus, 10-base template mRNA sequence around that cleavage site (ACACU:AAGAA) was chosen as the corresponding sequence of the test ssDNA oligomer.

Additions of the ssDNA oligomer resulted in chemical shift changes in a number of cross peaks in fast exchange mode on the NMR time scale (Fig. 5A). The spectral changes in fast exchange mode suggest that $K_d$ values for the interaction of HP0894 with these substrate homologues were relatively high. Fig. 5B shows the chemical shift changes of HP0894 residues when perturbed by the presence of the ssDNA oligomer (4 molar equivalents of HP0894). The residues with marked chemical shift changes are mapped on the HP0894 NMR structure in Fig. 5C. The HP0894 residues that are involved in nucleotide binding are clustered into three sites based on structure and sequence (Fig. 5C): site I, Lys$^8$ and Ser$^9$; site II, Lys$^{50}$, Gly$^{51}$, Lys$^{52}$, Trp$^{53}$, Lys$^{54}$, and Glu$^{58}$; and site III, Arg$^{80}$ (side-chain NH), His$^{84}$, Ser$^{85}$, Glu$^{86}$, Leu$^{87}$, and Phe$^{88}$. Overall, these three sites carry positive charges and form a triangle-shaped and concave active site on the three-dimensional NMR structure of HP0894.

To assess the sequence specificity of HP0894, chemical shift perturbations of $^{15}$N-labeled HP0894 were monitored with four molar equivalents of four kinds of synthetic 10-base ssDNA oligomers (ssDNA-U, ssDNA-A, ssDNA-C, and ssDNA-G), which only differed by the 5th nucleotide at their centers (Fig. 6). In our comparison of the chemical shift perturbations in each case, the residues Ser$^{85}$, Glu$^{86}$, Leu$^{87}$, Lys$^{52}$, and Trp$^{53}$ (side-chain NH) of HP0894 showed rather noticeable differences in chemical shift changes of HP0894 among the four ssDNA oligomers tested. Interestingly, the difference between ssDNA-U and ssDNA-C was larger than that between ssDNA-C and ssDNA-A despite the fact that dU and dC are both pyrimidine and structurally similar. In fact, the two-dimensional $[^{1}H-^{15}N]$-TROSY spectrum of $^{15}$N-labeled HP0894 mixed with ssDNA-A was nearly identical to that of HP0894-HP0895 Is a Toxin-Antitoxin System

FIGURE 3. Chemical shift perturbation mapping of the HP0895Ctp-binding region on HP0894. A, averaged chemical shift changes (ave) of individual residues of HP0894 upon binding with HP0895Ctp (1:1 molar ratio). The changes of the residues in obvious slow exchange mode are colored in red. B, ribbon and surface displays of HP0894 structure colored according to chemical shift perturbations. Residues showing obvious slow exchange mode chemical shift changes are in red (residues in the N-terminal region (N-ter)) or purple (residues in the C-terminal region (C-ter)). The program PyMOL (28) was used to visualize the structures.
DISCUSSION

After determining the solution structure of HP0894 (5), the crystal structure of E. coli YoeB, another RelE family toxin, was described (12). The YoeB monomer was found to share high structural similarity to HP0894 (Z score = 8.8, root mean square deviation = 2.9 Å) in a DALI-based analysis (30). This similarity raised the possibility that HP0894 might act as a TA toxin with intrinsic RNase activity. In addition, the NMR structure of a mutated construct (R83A/R85A) of E. coli RelE was reported recently (22), and that construct also had high similarity to that of HP0894 (Z score = 6.1, root mean square deviation = 3.0 Å).

In TA systems, the antitoxin gene is usually located directly upstream of the toxin gene, and on the H. pylori chromosome, the hp0895 gene is located directly upstream of the hp0894 gene (Fig. 1A). Therefore, we expected that if HP0894 is a TA toxin, then it would bind to HP0895. The 125-residue H. pylori-specific hypothetical protein HP0895 from H. pylori 26695 is a rather large protein when compared with other antitoxins (RelB, 79 residues; aRelB, 67 residues; YefM, 92 residues; and DinJ, 86 residues) and shares no detectable sequence similarity with those antitoxins. However, as revealed in the present study, HP0894 strongly binds to HP0895, with a 1:1 binding stoichiometry. HP0894 has RNase activity on mRNA, and HP0895 inhibits the decay of mRNA by HP0894. Moreover, HP0894 expression has a toxic effect on E. coli growth, but the co-expression of HP0895 neutralizes that HP0894 toxicity. Furthermore, His84 of HP0894, which is homologous to key catalytic residues of His83 of YoeB (12) and Arg85 of aRelE (7), is also essential for its RNase activity (Fig. 1D and supplemental Fig. S7) (33–35). In addition, HP0895 and HP0894–HP0895 complex bind to DNA fragments harboring their promoter regions. These results, including sequential and structural analyses, suggest that HP0894 is a RelE family toxin with intrinsic RNase activity and that HP0895 is its antitoxin.

Interestingly, in BLASTP (36) and BLAST2 SEQUENCE (37) analyses, the E. coli YafQ toxin shares detectable sequence similarity with HP0894 (identity, 33.0%; similarity, 54.9%), whereas E. coli RelE, E. coli YoeB, and P. horikoshii aRelE do not. In particular, in the region from nearly residue number 40 to the end, there is high sequence similarity between HP0894 and YafQ, and this region contains functionally important residues in HP0894, aRelE, RelE, and YoeB. A sequence alignment of HP0894, YafQ, RelE, aRelE, and YoeB, using the program ClustalW (32), also showed that HP0894 is similar to YafQ (supplemental Fig. S7). These findings indicate that HP0894 is a YafQ homologue. Therefore, we deduce that YafQ toxin also has intrinsic RNase activity, as observed with HP0894. Given that HP0894 shares high structural similarity with aRelE, RelE, and YoeB toxins (despite the low sequence similarity among them), it is reasonable to propose that the structure of the YafQ toxin is quite similar to that of HP0894.

Although HP0895 is a TA antitoxin, it has characteristics that are different from those of other antitoxins. First, unlike other antitoxins, which are generally acidic, HP0895 is a basic...
protein with a theoretical isoelectric point value of 9.11, which is confirmed by its higher solubility in acidic than basic buffer (data not shown). In addition, in the absence of HP0894 toxin and even in the HP0894-HP0895 complex, the HP0895 antitoxin exists as a relatively larger multimer or in an aggregated form rather than a dimer. In contrast, the antitoxins in the RelE-RelB (n/H11005), aRelE-aRelB (n/H11005), YoeB-YefM (n/H11005), and MazF-MazE (n/H11005) complexes form dimers (toxin-antitoxin2 (7, 12, 21, 38)). It has been suggested that the YafQ-DinJ complex comprises two toxin and two antitoxin molecules (39). Dimerization of antitoxins can be related to observations showing that the antitoxins regulate the TA operon by interacting with DNA as homodimers (7, 38, 40, 41). Based on those two differences, we suggest that the HP0895 antitoxin may interact with DNA in a manner different from those adopted by the other, above described antitoxins.

In the absence of toxins, native RelB, YefM, and aRelB antitoxins are regarded as being completely unfolded proteins or as having partially unfolded structures in their C-terminal regions (7, 8, 38, 41–44). Unfolded antitoxin structures have been reported to be related to their vulnerability to proteolytic degradation (7, 42, 43). Similarly, CD analyses on HP0895 and HP0895Ctp suggest that the unbound HP0895 antitoxin has a folded core and an unfolded C-terminal tail, as has been reported for RelB (38).
The comparison of HP0894 with its structural homologues indicated that some of catalytic residues involved in RNase activity in RNase Sa and YoeB are conserved in HP0894 (Fig. 7). For example, His<sup>85</sup> and Glu<sup>54</sup> of RNase Sa and His<sup>83</sup> and Glu<sup>46</sup> of YoeB are replaced with His<sup>84</sup> and Glu<sup>58</sup> in HP0894, respectively. However, Arg<sup>69</sup> of RNase Sa and Arg<sup>65</sup> of YoeB, which form catalytic triads with the above 2 residues, respectively, are not conserved (12, 44). However, Tyr<sup>84</sup> of YoeB, which was expected to be involved in base packing (12, 22), is conserved as Phe<sup>88</sup> in HP0894. NMR titration with RNA substrate homologues suggests that these HP0894 residues are involved in RNA substrate binding. Including these residues, the overall RNA-binding region is composed of three residue clusters. Among them, C-terminal residues Arg<sup>80</sup> and His<sup>84</sup>–Phe<sup>88</sup> in HP0894 seem to play critical roles in catalysis and RNA sequence specificity. In support of that suggestion, the C-terminal regions of YoeB and RelE are also essential for their toxicities or mRNA cleavage activity (12, 22). The residue region around Lys<sup>52</sup> (about Lys<sup>50</sup>–Lys<sup>54</sup>) is important for substrate binding and specific sequence recognition. The

**FIGURE 6. Differences in chemical shift perturbation patterns of HP0894 upon binding to ssDNA-U, -A, -C, and -G.** Superpositions of enlarged portions of two-dimensional [¹H-¹⁵N] TROSY spectra of HP0894 alone (black) and with added (1:4 molar ratio) ssDNA-U (red), -A (green), -C (blue), or -G (magenta) are shown. Relatively marked differences in chemical shift perturbations among ssDNA-U, -A, -C, and -G titrations were shown around Lys<sup>52</sup> and Trp<sup>53</sup> (side-chain NH) resonances (A) and around Ser<sup>85</sup>, Glu<sup>86</sup>, and Lys<sup>87</sup> resonances (B). Lines below the ssDNA sequences indicate specific (solid lines) or predicted (broken lines) sites recognized by the above residues in each case.
HP0894-HP0895 Is a Toxin-Antitoxin System

HP0894 Lys\(^8\)–Ser\(^9\) region is structurally dissimilar when compared with the active sites of RNase Sa or other RelE family toxins. However, according to \textit{in vitro} RNase activity and \textit{in vivo} toxicity tests with single mutated HP0894 (K8A or S9A) constructs, which showed that Lys\(^8\) and Ser\(^9\) residues are critical for RNase activity and toxicity of HP0894 (supplemental Fig. S8), this region is also important for mRNA binding.

With regard to these three binding regions, HP0894 has a differently shaped active catalytic site from those in RNase Sa or other RelE family toxins. Considering the overall arrangement of residues around the HP0894 active site, Arg\(^{80}\) of HP0894 seems to play a role corresponding to those of Arg\(^{69}\) in RNase Sa and Arg\(^{65}\) in YoeB.

Our results show that the cellular level functioning of HP0894 is inactivated by binding with HP0895. The direct interaction of these two proteins occurs mainly between the 30-residue C-terminal tail of HP0895 and the N-terminal secondary structure elements and an adjacent C-terminal \(\beta\)-strand of HP0894. Upon binding to HP0894, that unstructured C terminus tail of HP0895 seems to be transformed into structured form. Additionally, the interaction seems to cause conformational changes of HP0894 C-terminal residues such as His\(^{84}\) and Ser\(^{85}\). Similar interaction modes are seen in RelE-RelBc, and YoeB-YefM complexes, although both the core \(\beta\)-strand regions and the N-terminal regions of their toxins are involved in the RelE-RelBc and YoeB-YefM interactions (12, 22).

Our finding that HP0895 is a TA antitoxin introduces the possibility that the HP0894-HP0895 chromosomal TA system may be relevant in the pathogenicity of \textit{H. pylori}. A study by Graham \textit{et al.} (45) revealed that \textit{hp0895} is one of the \textit{H. pylori} genomic open reading frames that correspond to genes that are potentially expressed in response to interactions with the human gastric mucosa. This indicates that the HP0894-HP0895 TA system, especially through negative regulation of HP0894 toxin by the HP0895 antitoxin, may be related to the status of infections of \textit{H. pylori} in the human gastric mucosa and to its survival in that region.

It has been reported that drug resistance to \textit{H. pylori} has been increasing (46). Inhibition of the binding of antitoxin to toxin may enhance the toxic activity level of the toxin on cell growth; therefore, pathogen TA systems may be excellent targets for antibacterial agents. Thus, the HP0894-HP0895 couple may be an appropriate new target for antibacterial agents for \textit{H. pylori}. The information in this study about the binding aspect of these two proteins may be helpful in the design and development of new antibiotic drugs.

Acknowledgments—We thank the National Center for Inter-University Research Facilities (NCIRF) at Seoul National University and the Korea Basic Science Institute (KBSI) for providing high field NMR equipment.

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