Structural and Functional Characterization of Escherichia coli Toxin-Antitoxin Complex DinJ-YafQ*

**Yajing Liang**, **Zengqiang Gao**, **Fei Wang**, **Yangli Zhang**, **Yuhui Dong**,**1** and **Quansheng Liu**,**2**

From the **School of Life Sciences, University of Science and Technology of China, Hefei, Anhui Province 230027, China, the** §**Multidiscipline Research Center, Institute of High Energy Physics of the Chinese Academy of Sciences, 19B Yuequan Road, Beijing 100049, China, and the **Key Laboratory of Molecular Biology on Infectious Disease, Chongqing Medical University, YIXueYuanlu-1, Chongqing 400016, China**

**Background:** The type II TA dinJ-yafQ module autoregulates bacterial growth in response to environmental stimuli.

**Results:** The crystal structures of the DinJ-YafQ complex and free YafQ unveiled their structural details.

**Conclusion:** The DinJ-YafQ complex as a transcription repressor interacts with a defined region in its operator via the RHH domain of DinJ.

**Significance:** This study defines YafQ as a ribosome-dependent ribonuclease in vivo.

Toxin YafQ functions as a ribonuclease in the dinJ-yafQ toxin-antitoxin system of *Escherichia coli*. Antitoxin DinJ neutralizes YafQ-mediated toxicity by forming a stable protein complex. Here, crystal structures of the (DinJ)₂-(YafQ)₂ complex and the isolated YafQ toxin have been determined. The structure of the heterotetrameric complex (DinJ)₂-(YafQ)₂ revealed that the N-terminal region of DinJ folds into a ribbon-helix-helix motif and dimerizes for DNA recognition, and the C-terminal portion of each DinJ exclusively wraps around a YafQ molecule. Upon incorporation into the heterotetrameric complex, a conformational change of YafQ in close proximity to the catalytic site of the typical microbial ribonuclease fold was observed and validated. Mutagenesis experiments revealed that a DinJ mutant restored YafQ RNase activity in a tetramer complex in vitro but not in vivo. An electrophoretic mobility shift assay showed that one of the palindromic sequences present in the upstream intergenic region of DinJ served as a binding sequence for both the DinJ-YafQ complex and the antitoxin DinJ alone. Based on structure-guided and site-directed mutagenesis of DinJ-YafQ, we showed that two pairs of amino acids in DinJ were important for DNA binding: the R8A and K16A substitutions and the S31A and R35A substitutions in DinJ abolished the DNA binding ability of the DinJ-YafQ complex.

Located on plasmids or chromosomes, TA systems typically comprise two genes organized in an operon that codes both for a stable toxin and a labile antitoxin. Under normal cellular conditions, antitoxins typically interact with their cognate toxins to inhibit toxicity, allowing normal cell growth. However, under stress conditions, the expression of TA systems is decreased, leading to an imbalance in the amount of antitoxins and toxins present in the cell. Because toxins are more stable than antitoxins, toxins present in stressed cells are released, causing growth inhibition or even cell death.

Toxins are characterized as proteins, whereas antitoxins are either proteins or small noncoding RNAs. Currently, TA systems are classified into five classes (types I–V) according to the nature and mode of action of the antitoxin (9). In type I and III TA modules, the antitoxins are small noncoding RNAs (9). In type I TA systems, antisense RNAs act as antitoxins and inhibit translation of their cognate toxins by binding to the toxin-encoding mRNAs (10, 11). For example, the symR/symE module of *Escherichia coli* is a type I TA system (12). However, in type III TA systems, RNA antitoxins bind to the toxins directly. By forming a complex, RNA antitoxins neutralize a type III TA toxicity (10, 13, 14).

The only reported example of a type III TA system is the toxI/toxN module from *Pectobacterium carotovorum* (9, 13, 14). In type II TA systems, a protein antitoxin blocks the toxicity of a toxin by forming a stable complex (10). In type IV TA systems, the protein antitoxin cannot form a complex with its cognate toxin but acts as an antagonist for its toxicity (e.g. the yeeU/yeeV module from *E. coli*) (15). In type V TA systems, a protein antitoxin inhibits its cognate toxin by specifically cleaving its mRNA (e.g. the ghoS/ghoT module from *E. coli*) (16).

The type II TA systems are the best studied class of TA modules because they are abundant in bacterial genomes. In *E. coli* K12, at least 19 different type II TA systems have been identified on the chromosome and characterized (10), including relE-relB (17–21), dinJ-yafQ (7, 22, 23), yoeB-yefM (24, 25), chpBK-chpBI (26, 27), mazF-mazE (28–30), yafNO (31, 32), hipBA (33, 34), and hicAB (35). The TA system relBE is one of the best described TA modules in terms of regulation of activity and structural insights into the TA complex and its components.
Structure and Function of Toxin-Antitoxin Complex DinJ-YafQ

Although toxins generally exert their functions in crucial cellular processes, such as translation, DNA synthesis, cytoskeleton formation, membrane integrity, and cell wall biosynthesis, most of the characterized toxins, such as RelE, are endonucleases and inhibit translation by cleaving mRNAs with different specificity (10, 18). According to sequence similarities, the toxins YafQ, YoeB, HigB, and YhaV are classified within the RelE family (27, 36, 37). RelE, which contains a microbial RNase fold, is a ribosome-dependent RNase cleaving the mRNA codon positioned at the A-site in the ribosome, between the second and third nucleotides (38). However, antitoxin RelB wraps around RelE in the RelBE complex, thereby preventing entry of the toxin into the ribosome A-site and abolishing its toxicity (19).

In the E. coli dinJ-yafQ TA system, YafQ toxin, which also contains a microbial RNase fold, is an endoribonuclease that associates with the ribosome, and its overproduction causes growth inhibition or even cell death due to its RNA cleavage (7, 22). In vivo, YafQ selectively cleaves mRNA codons positioned in the A-site of the ribosome (7). Unlike RelE, YafQ exhibited robust ribosome-independent ribonuclease activity in vitro. Under normal growth conditions, antitoxin DinJ forms a stable complex with toxin YafQ, sequestering its incorporation into ribosomes and neutralizing its toxicity. However, stress-induced ATP-dependent proteases preferentially eliminate unstable DinJ, resulting in YafQ release and incorporation into ribosomes. DinJ and the DinJ-YafQ complex can autoregulate their expression through binding the upstream sequence of the dinJ-yafQ module (7, 10, 22). There are three imperfect palindromic sequences (palindromes pal I, pal I-II, and pal II), spanning the region −62 to −12 with respect to the translation start site of the dinJ-yafQ module of E. coli. They were previously reported as putative dinJ-yafQ operator regions (22). Palindromes pal I and II are juxtaposed with one base pair overlapping, whereas pal I-II comprises the 3′ region of pal I and the 5′ region of pal II. Pal II was previously shown to harbor a putative LexA box (7). Recent studies showed that only palindromes pal I and I-II are the response sites for autoregulation (22).

To date, the TA crystal structures of YoeB, YoeB-YefM, and MazF-MazE from E. coli and the RelE-RelB complex from E. coli and Pyrococcus horikoshii have been solved (17, 21, 25, 30). However, the structure of DinJ-YafQ has not yet been determined for any bacteria or archaea. In this study, we present the crystal structure of the DinJ-YafQ complex from E. coli at a resolution of 2.1 Å. The crystal structure revealed that DinJ consists of three domains: an N-terminal domain, a middle loop (linker), and a C-terminal domain. The N-terminal domain is mainly involved in homodimerization and also participates in DNA binding. The C-terminal domain of DinJ wraps around the globe-shaped YafQ. DinJ inhibits the toxicity of YafQ with the help of its intermediate loop covering the predicted active center of YafQ. Compared with the crystal structure of free YafQ, a conformational change in the positioning of the YafQ loop (α2-β3) in DinJ-YafQ in the vicinity of catalytic site was identified, which disrupts YafQ ribonucleolytic activity. By an electrophoretic mobility shift assay (EMSA), we also identified the shortest DNA fragment able to bind to the DinJ-YafQ complex. Based on the structure of DinJ-YafQ, we used site-directed mutagenesis of DinJ to determine which amino acids are important for DNA binding. The R8A and K16A substitutions and the S31A and R35A substitutions of the predicted binding site residues of DinJ abolished DNA binding, as shown by an EMSA.

EXPERIMENTAL PROCEDURES

Plasmids and Constructs—The open reading frame of the dinJ and yafQ genes were PCR-amplified from the E. coli BL21(DE3) strains. Restriction-digested PCR products were conjugated into pET28a or pCDFDuet-1 vector (Novagen) for generating construct pET28a-dinJ, pET28a-smt3-dinJ, and pCDF-smt3-YafQ. In detail, pET28a-dinJ encodes for DinJ without any hexa-His tag, pET28a-smt3-dinJ and pCDF-smt3-yafQ encode for DinJ and YafQ, respectively, with an N-terminal hexa-His tag and Smt3 fusion cleavable by Ulp1 (Table 1) (39). All constructs were confirmed by DNA sequencing.

Site-directed Mutagenesis of DinJ and YafQ—Plasmids for DinJ mutants (DinJ(R8A/K16A), DinJ(S31A/R35A), DinJ(R8A/K16A/S31A/R35A), DinJ(F49A), and DinJ(K159N/162N)) were obtained by site-directed mutagenesis with a template of pET28a-smt3-dinJ. Plasmids for YafQ mutants (YafQ(H87Q), YafQ(H23Q), and YafQ(K48A)) were obtained by site-directed mutagenesis with a template of pCDF-smt3-yafQ.

Protein Expression and Purification—See Table 1. To produce the DinJ-YafQ complex (or DinJ mutant complexes: DinJ(R8A/K16A)-YafQ, DinJ(S31A/R35A)-YafQ, DinJ(R8A/K16A/S31A/R35A)-YafQ, and DinJ(F49A)-YafQ, DinJ(K159N/162N)-YafQ), Rosetta2(DE3)plysS strain co-transformed with pCDF-smt3-yafQ and pET28a-smt3-dinJ (or a derivative, encoding for DinJ(R8A/K16A), DinJ(S31A/R35A), DinJ(R8A/K16A/S31A/R35A), DinJ(F49A), or DinJ(K159N/162N)) was utilized. To obtain DinJ, Rosetta2(DE3)plysS strain transformed with pET28a-smt3-dinJ was utilized. To obtain YafQ (H87Q), Rosetta2(DE3)plysS strain transformed with pCDF-smt3-yafQ was utilized. To obtain protein YafQ (or YafQ(H23Q) or YafQ(K48A)), Rosetta2(DE3)plysS strain co-transformed with pET28a-dinJ and pCDF-smt3-yafQ (or pCDF-smt3-yafQ (H23Q) or pCDF-smt3-yafQ (K48A)) was utilized.

The expression strain was grown in shaker flasks to approximately A600 0.6 at 37 °C and induced at 30 °C for 6 h with 0.2 mM isopropyl β-D-thiogalactopyranoside. After induction, cells were harvested, and pellets were flash-frozen. For performing protein purification, cell pellets were suspended in 50 mM Tris-HCl (pH 8.0), 20% (w/v) sucrose, 350 mM NaCl, 20 mM imidazole, 0.1% Tween 20, 1 mM PMSF, 1 mM β-mercaptoethanol, and 10 μg/ml DNase. Cell suspension was disrupted by a high-pressure homogenizer. Cell homogenate was centrifuged. Supernatant was subjected to nickel-nitrilotriacetic acid bead column purification. His-tagged protein or complex was eluted in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 250 mM imidazole, and 1 mM β-mercaptoethanol. Smt3 fusion proteins were cleaved by recombinant Ulp1 overnight at 4 °C and purified with HiTrap heparin HP and Superdex-75 16/60 chromatography (GE Healthcare). For purifying YafQ, mutants YafQ(H23Q) and YafQ(K48A), and the N-terminal hexa-His and Smt3 fusion of YafQ, mutants YafQ(H23Q) and YafQ(K48A), in complex with DinJ, were initially purified. They were then subjected to a
series of denaturation/renaturation steps, according to Ref. 7. Refolded Smt3-fused proteins were treated by Ulp1 digestion, and finally tag-removed YafQ and mutants were further purified with a Hitrap SP column and gel filtration chromatography (GE Healthcare). Protein fractions after chromatography were analyzed by SDS-PAGE.

**Dimethyl Pimelimidate Cross-linking of Protein DinJ**—Dimethyl pimelimidate (Sigma) was added to the reactions at a concentration of different protein in a binding buffer (5% glycerol, 10 mM Tris-Cl, pH 8.0, 5 mM MgCl₂, 100 mM NaCl, 10 mM β-mercaptoethanol). After incubation for 30 min, the resultant was electrophoresed on a 6% polyacrylamide gel with 0.25 TBE, and the gel was visualized by ethidium bromide staining.

**Total RNA Extraction and RNA Cleavage Analysis in Vitro**—Total cellular RNA was isolated from the E. coli DH5α strain, using the hot phenol extraction method (48). For RNA cleavage, total RNA was utilized for YafQ-mediated cleavage in vitro. The cleavage reaction (10 µl), containing 5 µg of total RNA and 0.5 µg of the YafQ or mutant protein, was incubated for 15 min at 37 °C in the reaction buffer (10 mM Tris-Cl, pH 8.0). The reaction was quenched by adding an equal volume of 2× loading dye buffer (New England Biolabs) and heated at 70 °C 3 min, prior to running on a 1% agarose gel. The agarose gel was visualized by ethidium bromide staining.

**RESULTS**

**Oligomerization States of DinJ, YafQ, and the DinJ-YafQ Complex**—To determine the oligomeric forms of recombinant DinJ, YafQ, and the DinJ-YafQ complex in solution, purified DinJ, mutant YafQ(H87Q), and DinJ-YafQ complex were pre-
Structure and Function of Toxin-Antitoxin Complex DinJ-YafQ

TABLE 2
Data collection and refinement statistics

|                              | Se-Met YafQ-DinJ | YafQ-DinJ native | Se-Met YafQ (H87Q) | WT YafQ | YafQ (H87Q) |
|------------------------------|------------------|------------------|--------------------|---------|-------------|
| Data collection              |                  |                  |                    |         |             |
| Wavelength (Å)               | 0.9791           | 1.0              | 0.9792             | 1.0     | 1.0         |
| Space group                  | C2               | C2               | 123                | C2221   | P1          |
| Unit-cell parameters         |                  |                  |                    |         |             |
| a = 175.31 Å, b = 119.79 Å, c = 119.72 Å, β = 130.11° | 2.10 (2.14-2.10) | 3.00 (3.05-1.00) | 1.50 (1.53-1.50) | 1.50 (1.53-1.50) |
| Resolution (Å)               | 2.50 (2.54-2.50) |                  |                    |         |             |
| Unique reflections           | 64.984 (1233)    | 111.791 (5575)   | 22.814 (1104)      | 39.681 (1853) |
| Completeness (%)             | 99.9% (100)      | 99.9% (100)      | 100% (100)         | 96.3% (92.9) |
| Redundancy                   | 7.5 (7.5)        | 4.9 (5.0)        | 10.0 (6.9)         | 3.9 (2.4) |
| Mean |                      | 45.41 (10.52)    | 32.98 (9.32)       | 27.21 (11.04) | 72.47 (16.23) | 45.35 (8.25) |
| Molecules in asymmetric unit | 16               | 16               | 2                  | 1       | 2           |
| Ramachandran plot (%)        |                  |                  |                    |         |             |
| Most favored                 | 99.56            | 98.89            | 98.88              |         |             |
| Allowed                      | 0.44             | 1.11             | 1.12               |         |             |
| Bond lengths (Å)             | 0.008            | 0.006            | 0.005              |         |             |
| Bond angles (degrees)        | 1.063            | 1.084            | 1.112              |         |             |

Values in parentheses are for the highest resolution shell.

Se-Met YafQ-DinJ heterodimers in the crystallographic asymmetric unit, consisting of four (DinJ)2-(YafQ)2 heterotetramers related to each other by noncrystallographic symmetries (Fig. 2A). The (DinJ)2-(YafQ)2 heterotetrameric form observed here was consistent with the oligomeric state described above, as determined by analytical gel filtration chromatography in solution (Fig. 1A). Each (DinJ)2-(YafQ)2 tetramer constitutes three globular domains as a triangular assembly: a DinJ homodimerization domain and two heterodimerization domains, each comprising a whole YafQ molecule and a DinJ C-terminal domain (Fig. 2A). In the resulting heterotetramer, the triangle-shaped structure with a YafQ-(DinJ)2-YafQ architecture of (DinJ)2-(YafQ)2 occupies an area of approximately 105 × 360 Å. The final structure covers most of YafQ (residues 3–92 of 92) and DinJ (residues 3–86 of 86).

In the crystal structure of the complex, the YafQ monomer forms a compact globular structure with an α/β/α-fold. This protein consists of a central four-stranded β-sheet (β1–β4) core surrounded by four α-helices, denoted α1–α4. Together with the β-sheet core, helix α4 and the loop connecting helix α3 and strand β2 form a cleft. Notably, one sulfate ion (SO$_4^-$) is embedded in the cleft (Fig. 2B) coordinated by residue His-50, Asp-61, His-63, and His-87 of YafQ via hydrogen bonds. This cleft possibly represents the catalytic site for YafQ. The catalytic site of the toxin YafQ mostly harbors aromatic or hydrophobic residues that are thought to accommodate the substrate mRNA. In contrast, the DinJ monomer resumes a more elongated shape in the complex crystal structure, with the topology β1, α1, α2, α3, β2, and α4. This elongated structure can be divided into three portions: an N-terminal domain (β1–α1–α2),...
YafQ may be interesting and may contribute to blocking potential RNA substrate access to the catalytic cavity (Fig. 2B; see below).

Crystal Structure of Isolated YafQ and YafQ(H87Q) Mutant—To further assess the functional mechanism behind ribonuclease catalysis and toxicity, the structures of WT YafQ of *E. coli* and its mutant YafQ(H87Q) were determined (for related structural details, see Table 2). The crystal structures of WT YafQ and mutant YafQ(H87Q) in isolation were almost identical except for the mutated residue (Fig. 2C). When overlaid onto the crystal structure of the DinJ-YafQ complex, the crystal structure of WT YafQ superimposed well, except at one site (see below), the loop connecting helix α3 and strand β2 (loop α3-β2, a switch loop), which appears to rearrange into a closed and compressed configuration in isolated YafQ. Loop α3-β2 in the isolated YafQ swings toward the β-sheet core, narrowing the passage that would accommodate the interaction of DinJ helix α3 in the TA complex. The positioning of Trp-56 in the α3-β2 loop alters most dramatically.

**Possible Functional Site of YafQ and YafQ Ribonuclease Activity in Vitro**—As a RelE family member, the structure of YafQ contains a microbial RNase fold that more closely resembles RNase T1 from *Aspergillus oryzae* (Fig. 2D) than the RelE and YoeB structures. We superimposed the structure of RNase T1 (Protein Data Bank entry 1B2M) from *A. oryzae* in complex with GpU with the isolated YafQ structure (Fig. 2D). We noticed that, despite a lack of sequence homology between YafQ and RNase T1, key residues at the active site were highly conserved. In RNase T1, the active site is composed of residues Tyr-38, His-40, Glu-58, Arg-77, His-92, and Phe-100 (Fig. 2D) (49). After superimposing the YafQ structure, His-50, Asp-61, His-87, and Phe-91 of YafQ overlapped with His-40, Glu-58, His-92, and Phe-100 of RNase T1, respectively. However, residues Tyr-38 and Arg-77 of RNase T1 were substituted by Lys-48 and Ile-69, respectively, in the YafQ structure (Fig. 2D). His-63 in the neighboring β strand of YafQ might play a role similar to that of Arg-77 of RNase T1, although when their structures are superimposed, Ile-69 of YafQ is equivalent to Arg-77 of RNase T1. Catalysis of YafQ may proceed via a mechanism proposed for RNase T1, whereby a protonated His-87 and an unprotonated Asp-61 in YafQ probably constitute the catalytic acid-base pair. However, mutation analysis indicated that the identity of residue Asp-61 in YafQ was not essential for cell viability. It is possible that His-50 of YafQ, which is replaced by Glu-46 in YoeB, matches with His-87 as the catalytic acid-base pair. Alternatively, as proposed for YoeB, it is likely that base A1493 of 16 S rRNA might act as a general base in the absence of Asp-61 *in vivo* (50). Both YafQ and YoeB, retaining a complete set of catalytic residues for the RNase fold, are capable of cleaving certain RNAs *in vitro*, whereas RelE is not. However, *in vivo*, the role of ribosome recruiting in a RelE family toxin at its A site may therefore be to stabilize mRNA in a conformation that facilitates cleavage (38).

In YafQ crystal structures, an embedded sulfate ion (SO₄²⁻) in isolated YafQ, or SO₄⁻₁ in complex) is coordinated by residues His-50, Asp-61, His-63, and His-87 in the putative catalytic cleft. This sulfate is believed to mimic the scissile phosphate of substrate RNA. Modeling the nucleotide GpU in the
RNase T1 structure into the active site of isolated YafQ shows that the phosphate of 3'-GMP overlaps well with SO4-A. Additionally, a second sulfate, SO4-B, in the isolated YafQ structure is 7.1 Å away from SO4-A. The proximity of these two sulfates might imply how an RNA substrate is situated over the YafQ active site in the process of cleavage.

To test YafQ ribonuclease activity in vitro, we assayed recombinant YafQ using total cellular RNAs extracted from E. coli strain DH5α as substrate. Wild-type YafQ was purified from the DinJ-YafQ complex through a series of denaturation/renaturation steps. The results showed that WT YafQ efficiently cleaved DH5α total RNA in a salt-sensitive manner, but the DinJ-YafQ complex and mutant YafQ(H87Q) failed to cleave DH5α total RNA. Mutant YafQ(H87Q) retained the interaction with DinJ to form the heterotetramer. We further characterized mutants YafQ(K48A) (equivalent to Lys-44 of YoeB) and YafQ(H23Q). Based on structural analysis, residue Lys-48 of YafQ was found to be in close proximity to the catalytic site and 3 Å away from the second sulfate SO4-B in the YafQ crystal structure (Fig. 2D), indicating that this residue is likely to be important in catalysis or RNA binding. Residue His-23 may also play a role in catalysis in the YafQ dimeric form, as observed with the YafQ crystal. In the free YafQ structure, residue His-23 was close to the active site of a neighboring YafQ in the crystal packing. However, an in vitro assay for ribonuclease activity showed that in both the K48A and H23Q mutants, a moderate decrease in YafQ ribonuclease activity occurred (Fig. 3A).

**Hydrophobic Interaction between DinJ Helix α3 and YafQ**

Contributes to DinJ Antidote Suppressing YafQ Ribonuclease Activity—In the DinJ-YafQ complex structure, YafQ toxicity was blocked due to its interaction with antidote DinJ, and YafQ toxicity is known to be caused by its RNase activity. The YafQ RNase catalytic region is covered by the loop linker and helix α3 of DinJ (Figs. 2B and 3B). The hydrophobic interactions between DinJ helix α3 and YafQ expel loop α3-β2 of YafQ from the β-sheet core, leading to a local conformational change in YafQ, compared with the structure of free YafQ. The positioning of Trp-56 in the α3-β2 loop alters most dramatically. This change in YafQ might interfere with its ribonuclease activity and toxicity. Next, we investigated whether any perturbations of these interacting interfaces could release YafQ RNase activ-
ity. Two DinJ mutants were constructed (F49A and I59N/I62N), and their interaction with YafQ was analyzed. The side chain of residue Phe-49 in the loop linker of DinJ interacts with His-50 of YafQ via a $\pi-\pi$ interaction (Fig. 2B). Residues Ile-59 and Ile-62 located in helix 3 of DinJ directly interact with YafQ by a hydrophobic interaction (Fig. 3C). The growth of both mutant strains was unaffected, indicating that the DinJ variants neutralized YafQ toxicity similarly to WT in vivo. It is probable that, in vivo, YafQ was still capable of binding to these DinJ mutants but could not incorporate ribosomes and therefore lost its ribosome-dependent RNase activity. Indeed, in vitro, both the DinJ(F49A)-YafQ and DinJ(I59N/I62N)-YafQ mutant complex could be co-purified by gel filtration chromatography, demonstrating that the interaction between $\beta 2$ and $\alpha 4$ of DinJ (without the contribution of $\alpha 3$) and YafQ is sufficient for maintaining a complex assembly. Strikingly, in vitro, the mutant DinJ(I59N/I62N)-YafQ complex cleaved RNA similarly to isolated YafQ. Mutation of residue Phe-49 to Ala in DinJ did not release YafQ from its complex with DinJ, and the RNase function of YafQ was not activated (Fig. 3B). Notably, both mutant complexes were still able to bind a DNA target (Fig. 3D).

Both the DinJ-YafQ Complex and DinJ Alone Only Bind One of the Three Imperfect Palindromic Sequences in the dinJ-yafQ Operator Region—In the dinJ-yafQ system, three imperfect palindromic sequences (palindromes pal I, pal I-II, and pal II) have been reported previously as putative dinJ-yafQ operator regions (22). Palindromes pal I and II are juxtaposed with one base pair overlapping, whereas pal I-II comprises the 3' region of pal I and the 5' region part of pal II (Fig. 4A). To validate which palindrome with inverted repeats is the operator region responsible for autorepression regulated by the DinJ-YafQ complex, these three sequences were tested with purified DinJ-YafQ in an EMSA. An intergenic fragment of dinJ-yafQ was included as a control (22). Within the protein concentration range 1–10 $\mu$M, pal I (CGCTGTTGCTCATTTGAGCTACATT) (reverted repeat underlined) and pal I-II (TTTGAGCTACAATTCAAGCTGAATAA) bound to the DinJ-YafQ complex, generating distinct upshifts in the observed bands (Fig. 4B, lane 5 and 6), with a migration pattern analogous to the dinJ-yafQ intergenic fragment control. Pal II (TTTGGAGCTACAATTCAAGCTGAATAA) bound to DinJ-YafQ more specifically than pal I (Fig. 4, C and D). However, pal II, harboring a putative LexA box, did not bind to the DinJ-YafQ complex.
complex (only a smear was evident on the gel) (Fig. 4B, lane 7). These results were consistent with an earlier report that demonstrated pal I and pal I-II to be DinJ-YafQ binding fragments (22). To precisely locate the boundaries of the binding sites and define the consensus sequences in pal I and pal I-II for DinJ-YafQ binding, a more comprehensive gel shift analysis was undertaken using gradually shortened DNA duplexes. We synthesized a series of deletion duplexes of pal I-II, and investigated their binding ability to DinJ-YafQ by EMSA. The shortest DNA duplex, which was 11 bp in length (I-II-12, TTTGAGCTACA), was located at the left palindromic sequence in pal I-II and was able to bind to the DinJ-YafQ complex (Fig. 5, A and B). Surprisingly, we were unable to obtain any fragments shortened from the 5′-end of pal I-II that were able to bind DinJ-YafQ. Even one duplex, which was only shortened by one base pair at the 5′-end of pal I-II (I-II-4), showed a significant loss in affinity to the DinJ-YafQ complex (compared with I-II-7), suggesting the importance of this 5′ extremity for binding. However, we found that the T-A base pair at the 5′-end of pal I-II-7 can be substituted to a C-G base pair (IId7G) with only a slight reduction in binding (Fig. 5C). Other base pairs in this 5′ region did not contribute significantly to binding affinity. Taken together, our findings indicate that the specific binding site for DinJ-YafQ is located in the 11 bp at the 5′-end of pal I-II, with a consensus sequence of TTTGAGCTACA.

**FIGURE 4. DinJ and DinJ-YafQ binding to the putative dinj–yafQ operator in vitro.** A, schematic of the dinj–yafQ upstream region. It spans from its translation start site (SS) 0 to −69, including three overlapping palindromes: pal I (in boldface type), pal I-II (partially in boldface type), and pal II. Underlines indicate axes of symmetry of repeated sequences, and highlights in gray indicate the −10 box and −35 box in the promoter. Half-sites are boxed. B, EMSAs demonstrated that protein DinJ (35 μM), YafQ (35 μM), and the DinJ–YafQ complex (10 μM) binds to pal I, I-II, and II (0.5 μM), respectively. C, a comparison of DinJ (top) and DinJ-YafQ (bottom) binding ability to pal I by EMSA. D, a comparison of DinJ (top) and DinJ-YafQ (bottom) binding ability to pal I-II by EMSA. The concentration of DNA duplex was constant at 0.5 μM in all EMSAs. DinJ and DinJ-YafQ complex concentrations (0, 1, 2, 4, 5, 6, 8, 10, 15, and 20 μM) were titrated in C, and DinJ and DinJ-YafQ complex concentrations (0, 1, 5, and 10 μM) were titrated in D.
Because pal I-II partially overlaps with pal I and they share a common sequence of pal I-II-10 (TTTGAGCTACAATT) (Fig. 4A), we next reinvestigated the binding sites in the context of pal I. We questioned whether pal I might provide an additional binding site for DinJ-YafQ, because we occasionally observed two shifted bands at certain concentrations of DinJ-YafQ in EMSAs with pal I (Fig. 4B). Indeed, EMSA with a series of deletion duplexes of pal I showed that one additional deletion duplex, I-3, retained binding ability to DinJ-YafQ, generating a single shifted band (Fig. 5D). This sequence of duplex, I-3 (CGCTGTTGCTCAATT), located at the left half-site side of pal I, bears an imperfect reverted sequence of pal I-II-10 (TTTGAGCTACAATT). Therefore, pal I harbors two half-sites in the dinJ-yafQ operator for DinJ-YafQ regulation, and each of the individual pal I half-sites (I-3 and I-II-10) was able to bind to DinJ-YafQ alone. This explains the observation that pal I-II was also capable of interacting with the DinJ-YafQ complex because pal I-II contains one half-site overlapped by pal I.

The crystal structure of DinJ revealed that it contained an RHH motif in dimeric form in its N-terminal region. This RHH fold is thought to be responsible for binding to each half-site in a palindrome. Next, we addressed whether DNA binding sites are solely recognized by DinJ. We performed similar experiments with all three palindromic candidates. Surprisingly, DinJ was able to bind to pal I and the whole intergenic fragment, but it was unable to bind to both pal I-II and pal II (Fig. 4B). We deduced that pal I contains two half-sites for DinJ-YafQ binding and that DinJ probably requires the presence of two half-sites in pal I simultaneously for binding. The affinity of a half-site present in pal I-II with DinJ dimer was relatively low; however, a pal I fragment with two half-sites might have a higher affinity for binding DinJ when a dimer-of-dimer DinJ forms. This would be consistent with the observation that DinJ formed a homotrimer in solution. As noted, in contrast to the DinJ-YafQ complex which bound each half-site in the operator separately, DinJ could not bind to a DNA duplex harboring only
one palindromic half-site (pal I-II-10 or I-3) in our hands (Fig. 4, A and D) (data not shown). It is probable that YafQ increases the binding affinity of DinJ (in the DinJ-YafQ complex) to individual half-sites in pal I. Overall, we deduced that pal I (positioned at +62 to +37 from the translation start site) is the consensus sequence of the dinJ-yafQ operator.

**DISCUSSION**

In this report, we determined the crystal structure of YafQ in complex with DinJ and the crystal structure of free YafQ. Both structures revealed that YafQ resembles a microbial RNase fold. According to structure-based alignment, all critical residues constituting the enzymatic site of YafQ were highly conserved with those of RNase T1. A recent mutagenesis study on the ribonuclease activity of YafQ demonstrated that His-50, His-63, Asp-67, Arg-83, His-87, and Phe-91 substitutions of the predicted active site residues of YafQ abolished mRNA cleavage *in vivo*, whereas Asp-61 and Phe-91 mutations inhibited YafQ ribonuclease activity only moderately. This is in agreement with the results revealed by the crystal structure of YafQ. As an endoribonuclease, YafQ enzymatic activity is blocked upon the formation a DinJ-YafQ complex. The coiled linker and helix H3 of DinJ in the DinJ-YafQ complex might play critical roles in blockage of RNA access to the catalytic site. Our site-directed mutagenesis studies showed that mutations in helix H3 of DinJ conferred ribonuclease activity of the DinJ-YafQ complex. This is in agreement with our expectations that the helix H3 mutant of DinJ designed to disrupt the hydrophobic interactions with YafQ would lead to constriction of the Trp-56 loop in YafQ back to the H-sheet core of YafQ. Intriguingly, site mutations of the coiled linker region or helix H3 of DinJ did not change the formation of the DinJ-YafQ complex, indicating that the primary interactions between DinJ and YafQ were mainly attributed to the C-terminal portion of DinJ. This interaction was shown to prevent YafQ binding to the ribosome and thereby inhibit its ribosome-dependent ribonuclease activity and toxicity *in vivo*.

Regarding the three imperfect inverted repeat sequences (pal I, pal I-II, and pal II), which overlap in the dinJ-yafQ system, we verified that pal I is the operator for regulating the DinJ-YafQ TA system. DinJ and YafQ assemble into a heterotetramer (YafQ-(DinJ)2)-YafQ capable of binding to duplex pal I. DinJ alone was also able to bind to pal I, but it required the presence of...
of two imperfect inverted repeats in the pal I sequence for effective binding. These findings raise the question of what role pal I-II and pal II are playing in transcriptional regulation of dinJ-yafQ and whether they are specific cis-elements that are recognized by other regulatory factors. For example, pal II might be LexA-regulated in the SOS response. Pal I-II might also be recognized by other unidentified regulatory factors for modulating dinJ-yafQ expression, and there may be other TA factors involved in cross-regulation (51). It would therefore be interesting to investigate the possibility of a coordination network for global TA system regulation. Future work may enhance our understanding in this area.

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