In Vitro Selection of RNA Aptamers that Bind to Colicin E3 and Structurally Resemble the Decoding Site of 16S Ribosomal RNA†

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ABSTRACT: Colicin E3 is a ribonuclease that specifically cleaves at the site after A1493 of 16S rRNA in *Escherichia coli* ribosomes, thus inactivating translation. To analyze the interaction between colicin E3 and 16S rRNA, we used *in vitro* selection to isolate RNA ligands (aptamers) that bind to the C-terminal ribonuclease domain of colicin E3, from a degenerate RNA pool. Although the aptamers were not digested by colicin E3, they specifically bound to the protein (K_d = 2−14 nM) and prevented the 16S rRNA cleavage by the C-terminal ribonuclease domain. Among these aptamers, aptamer F2-1 has a sequence similar to that of the region around the cleavage site from residue 1484 to 1506, including the decoding site, of *E. coli* 16S rRNA. The secondary structure of aptamer F2-1 was determined by the base pair covariation among the variants obtained by a second *in vitro* selection, using a doped RNA pool based on the aptamer F2-1 sequence. The sequence and structural similarities between the aptamers and 16S rRNA provide insights into the recognition of colicin E3 by this specific 16S rRNA region.

Colicin E3, an antibacterial protein, is encoded by plasmids within certain *Escherichia coli* strains and kills sensitive *E. coli* cells. This cytotoxic activity of colicin E3 is associated with the 11.8 kDa C-terminal ribonuclease domain (CRD) of the protein (*1*−*4*). The *E. coli* strains that produce colicin E3 resist the toxicity, because colicin E3 is expressed as a tight 1:1 complex with an immunity protein (*5*−*9*). The interactions between colicin E3 and the sensitive *E. coli* cells proceed in three steps: (i) binding of colicin E3 to a surface receptor, BtuB, on the outer bacterial membrane, (ii) translocation of colicin E3 through the cell envelope, and (iii) cleavage at the position between adenine 1493 and guanine 1494 of 16S rRNA in ribosomes by the CRD, thereby inactivating translation.

The colicin E3 cleavage site is close to the 3′ end of 16S rRNA and lies within the decoding region, which reportedly functions in the fidelity of protein synthesis by interacting with both the mRNA and tRNA located in the A site of ribosomes (*10*). The decoding region interacts with various molecules, such as initiation factors 1 and 3 and aminoglycoside antibiotics. The initiation factors required for the translation initiation in *E. coli* (*11*−*13*), and the binding of certain aminoglycoside antibiotics, such as neomycin, kanamycin, and paromomycin, changes the structure of the decoding region, inducing codon misreading in translation (*14*−*17*). Initiation complex formation on ribosomes with the initiation factors is impaired by treatments with colicin E3 (*18*). On the other hand, the specific cleavage by colicin E3 is prevented by the binding of aminoglycoside antibiotics to the decoding region (*19*). Thus, all of these molecules interact with the decoding region, and they drastically change the ribosome function by their actions in the 16S rRNA decoding region.

To illuminate the interaction between colicin E3 and 16S rRNA, the specific cleavage has been examined under several

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different conditions. The colicin E3 CRD can be isolated as a stably folded, active protein fragment, which cleaves 16S rRNA *in vitro* (*20*). Specific cleavage of 16S rRNA was observed when either 30S subunits or 70S ribosomes were incubated with the CRD (*20*−*23*), but nonspecific cleavage occurred when 16S rRNA isolated from ribosomes was used (*24*). These observations suggest that a certain 16S rRNA tertiary structure and/or the association of ribosomal proteins may be required for the specific cleavage. A recently determined model structure of the complex of the ribosomal 30S subunit and colicin E3, obtained via docking of both crystal structures, predicted that several residues in the 16S rRNA and ribosomal proteins interact with colicin E3 (*25*, *26*). However, the actual recognition mode between 16S rRNA and colicin E3 has not been identified.

To analyze the interaction between colicin E3 and 16S rRNA, we isolated artificial RNA ligands (aptamers) that bind to the colicin E3 CRD. *In vitro* selections of RNA aptamers targeted to RNA-binding proteins have often yielded motifs similar to those of the natural RNAs, and have provided valuable information about the protein−RNA interactions (*27*−*31*). In contrast, aptamers targeting proteins related to the cleavage of specific RNAs rarely bear a resemblance to the natural RNAs, but inhibit the protein activity in general (*32*, *33*). Nevertheless, we obtained aptamers that not only bind to the colicin E3 CRD and inhibit its activity but also share sequence and structural resemblance with the region around the 16S rRNA cleavage site. Here, we describe the *in vitro* selection and the structural features of the anti-colicin E3 aptamers.

EXPERIMENTAL PROCEDURES

Protein Preparations. The colicin E3, E4, and E6 CRDs were prepared according to literature methods (*7*, *34*, *35*). The DNA fragment encoding both the 110 C-terminal amino acids of colicin E3 and the E3 immunity protein was recloned from plasmid ColE3-CA38 and replaced with *colE3-immE3* genes under the initiation codon of *colE3* of plasmid pSH357 (*7*) as described in the literature (*34*). The homologous DNA fragments of ColE4-CT9 and ColE6-CT14 were used for the preparation of two heterodimer colicin E4 CRD (111 amino acids)−E4 immunity protein and colicin E6 CRD (110 amino acids)−E6 immunity protein complexes, respectively (*35*). The colicin E3 CRD and the E3 immunity protein, and those for colicins E4 and E6, were purified from the their heterodimer complex by the treatment with 6 M urea as described for colicin E5 (*34*).

Preparation of the Random RNA Pool. A 140-mer oligonucleotide [5′-GGTAATACGACTCACTATAGGGAGAATTCCGACCAGAAGCTT-(N72)-CATATGTGCGTCTACATGGATCCTCA-3′], consisting of 72 random nucleotides flanked by 39 and 24 nucleotide PCR primer binding sites (*36*), was synthesized on an Applied Biosystems (Foster City, CA) model 392 DNA/RNA synthesizer. The double-stranded DNA pool was prepared by PCR amplification of the 140-mer single-stranded N72 DNA pool (approximately 1.3×10^{13} sequences) using forward primer 39.72 (5′-GGTAATACGACTCACTATAGGGAGAATTCCGACCAGAAG-3′) and reverse primer 24.72 (5′-TGAGGATCCATGTAGACGCACATA-3′). A random RNA pool was transcribed from the amplified DNA pool by T7 RNA polymerase, using an AmpliScribe kit (Epicentre Technologies, Madison, WI). Transcripts were purified on a 10% polyacrylamide/7 M urea gel.

In Vitro Selection. The RNA pool (121-mer) was folded by heating at 75 °C for 3 min and cooling to room temperature in binding buffer [10 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 30 mM NH_4Cl]. While 1.7 μM RNA was used in the first round, the RNA concentration was gradually reduced to 0.05 μM in the twelfth round. The initial selection involved 500 pmol of RNA (approximately 23 copies of each sequence). To remove the RNA that was nonspecifically bound to colicin E3, an *E. coli* tRNA mixture (5 μM) (Sigma Chemical Co., St. Louis, MO) was added to the binding reaction mixture in the second through fifth rounds.

Prior to the reaction, the RNA was filtered through a nitrocellulose filter (HAWP filters, Millipore, Bedford, MA), and this prefiltration step was repeated from two to three times to remove the filter-binding RNAs. The RNA was incubated with the colicin E3 CRD at 37 °C for 60 min. The binding reaction mixture was filtered through the nitrocellulose filter under low vacuum, followed by washing with binding buffer for removal of the nonspecifically or weakly bound species. The RNAs on the filter were eluted twice with 200 μL of an elution buffer [7 M urea, 0.1 M sodium citrate (pH 5.0) and 3 mM EDTA (pH 8.0)] at 100 °C for 5 min. The eluted RNAs were precipitated with isopropyl alcohol. The selected RNAs were reverse-transcribed into DNA and amplified by PCR, using a One Step RNA PCR Kit with AMV reverse transcriptase and Taq polymerase (TaKaRa, Shiga), to generate templates for the next round of T7 *in vitro* transcription. During the sixth to twelfth rounds, the RNAs were passed over the nitrocellulose filter prior to amplification, to remove the RNA species that bound to the filter (*30*).

Nitrocellulose Filter Binding Assay. To assay pool binding, 0.1 μM 5′-^{32}P-labeled RNA was incubated with the colicin E3 CRD (0.1 μM), in binding buffer (100 μL) containing the *E. coli* tRNA mixture (5 μM) at 37 °C for 60 min. A portion (90 μL) of the binding reaction mixture was applied to a prewetted nitrocellulose filter under low vacuum, and the filter was washed twice with 200 μL of binding buffer. The retained counts were quantitated using a Bio-imaging analyzer (Fuji model BAS 2500) and were compared to the applied counts.

Determination of Colicin E3 Binding Dissociation Constants (K_d's). RNAs were prepared from all selected clones, for which sequencing of mini-prep DNA indicated a full-length, single-insert cDNA. Each 5′-^{32}P-labeled RNA (0.6 nM) was incubated with various concentrations (0.05−300 nM) of the colicin E3 CRD in binding buffer (100 μL) at 37 °C for 60 min. Although the binding of most aptamers (K_d < 15 nM) to the colicin E3 CRD reached equilibrium in 5 min, for the K_d determination of the aptamers with lower K_d values, the binding assay was performed for 60 min. The RNA was assayed in the same manner as in the pool binding assay, except that 45 μL of the binding reaction mixture was applied to the nitrocellulose filter. The K_d values were calculated by using Kaleidagraph (Abelbeck Software, Reading, PA) (*37*).

Cloning and Sequencing. Aptamers from the twelfth round of selection were converted into double-stranded DNA and cloned into the TA cloning vector (Invitrogen, San Diego,

CA). The plasmid DNA was sequenced by the dye terminator method (Applied Biosystems) on an automatic sequencer (model 377, Applied Biosystems).

Poly(U)-Dependent Polyphenylalanine Synthesis in the Presence of the Colicin E3 CRD and the Anti-Colicin E3 Aptamer. *E. coli* tRNA ($tRNA^{mix}_{E.coli}$) was purchased from Boehringer. Polyuridylic acid [poly(U)], pyruvate kinase, phosphoenolpyruvate, ATP, and GTP were purchased from Sigma, and L-[^{14}C]phenylalanine was from Amersham. The cell-free extract was prepared from *E. coli* strain A19 (*38*). Cells were grown at 37 °C in LB broth and were harvested in mid-log phase. The 105000*g* supernatant (S100) was purified by DEAE DE-52 chromatography, to remove the nucleic acids. Ribosomes were obtained as an S100 precipitate.

The colicin E3 CRD (1.25 pmol) was pretreated with 0−1250 pmol of anti-colicin E3 aptamers, in a solution (16 μL) containing 10 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 30 mM NH_4Cl, for 15 min at 37 °C. The CRD solution was mixed with ribosomes (20 pmol) and was incubated at 37 °C for 5 min. The solution was added to the S100 fraction (5.0 A_{280}/mL) containing $tRNA^{mix}_{E.coli}$ (5 mg/mL), 1 mM ATP, 0.2 mM GTP, pyruvate kinase (30 μg/mL), 5 mM phosphoenolpyruvate, polyuridylic acid (1 mg/mL), and 0.1 mM L-[^{14}C]phenylalanine. The mixture (50 μL) was incubated for 15 min. The polyphenylalanine synthesis was quenched with 10% TCA, and the phenylalanyl-tRNA was degraded with hot TCA. The radioactivity of the TCA-insoluble fraction was measured with a liquid scintillation counter (Packard TRI-CARB 2000). The efficiency of polyphenylalanine synthesis in the presence of the colicin E3 CRD treated with each aptamer was compared to that without the CRD.

Pool Construction for the Reselection of Aptamer F2-1. A doped DNA pool, with a sequence based on that of aptamer F2-1, was synthesized. Phosphoramidite mixtures were used to introduce point mutations at a rate of 50% per position. The double-stranded DNA pool and the subsequent RNA pool were prepared in the same manner as described above (Preparation of the Random RNA Pool). Although the random region of the original pool comprises 72 nucleotides, aptamer F2-1 has a 70-nucleotide random region, due to the deletion of two nucleotides during the preceding selection.

In Vitro Reselection from the Doped Pool of Aptamer F2-1. The purified RNA pool (119-mer) was used for the reselection. The RNA pool was denatured and annealed. Since BSA was present in the colicin E3 stock solution, BSA-binding and filter-binding RNAs were removed by prefiltration. The RNA was preincubated with 1.3 fM BSA at 37 °C for 25 min, and then was passed through the nitrocellulose filter. The RNA was incubated with colicin E3 (200 pmol) in binding buffer (1200 μL) at 37 °C for 60 min. In the first to third rounds of selection, 2000 pmol of RNA was used, followed by 500 pmol of RNA in the fourth to sixth rounds, and 200 pmol of RNA in the seventh and eighth rounds. Subsequent procedures were the same as those described above (*In Vitro* Selection).

CMCT Modification Interference Experiments for Aptamer F2-1. The experiments were carried out according to a previously described method (*39*). The renatured aptamer F2-1 (5 pmol) was incubated with or without colicin E3 (50 pmol) in modification buffer (10 μL), containing 10 mM Hepes-KOH (pH 7.8), 10 mM $MgCl_2$, and 30 mM KCl, for 30 min at 37 °C. The RNA or RNA−colicin E3 complex was incubated with or without 10 μL of CMCT [1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate, 42 mg/mL in modification buffer] for 40 min at 30 °C. The CMCT reactions were terminated by adding 0.3 M NaOAc (pH 6.0, 200 μL) and the mixtures precipitated. After centrifugation, the pellets were resuspended in 0.3 M NaOAc (pH 6.0) and extracted with phenol and chloroform. The RNAs were precipitated and dissolved in 10 μL of dH_2O (final RNA concentration of 0.5 pmol/μL). The chemical modifications were monitored by a primer extension analysis on aptamer F2-1, using AMV reverse transcriptase XL (TaKaRa) and 5′-^{32}P-labeled reverse primer 24.72. The cDNA products of the primer extension reactions were separated by 10% polyacrylamide gel electrophoresis. The positions of the stops in cDNA synthesis were visualized on a gel autoradiogram and identified by reference to a dideoxy A sequencing reaction of aptamer F2-1.

RESULTS AND DISCUSSION

In Vitro Selection. To isolate RNA aptamers that specifically interact with the catalytic domain of the protein, we used the colicin E3 CRD as a selection target, because it has full activity for the specific cleavage of 16S rRNA in ribosomes (*20*). *In vitro* selection was initiated with an RNA pool (3.0×10^{14} molecules) that contained 72 randomized positions. We chose a relatively long random region to find characteristic sequences at various positions in the region. The binding of the RNA species to the colicin E3 CRD was carried out under conditions similar to those used for the cleavage of 16S rRNA by colicin E3 (*1*). In each round of selection, the CRD was mixed with the RNA pool, and the bound species were separated from the unbound species by passing the mixture over a modified cellulose filter. The selection conditions and the activities of binding to the protein and the filter are summarized in Table 1 (N72). To gradually increase the selection stringency, the concentrations of both the RNA pool and the protein were occasionally reduced in the subsequent rounds. During rounds 2−5, excess amounts of *E. coli* tRNA mixtures were added to the protein/RNA solution, to eliminate the RNA species that nonspecifically bound to the CRD. During rounds 6−12, to remove the filter-binding species from the pool, postfiltration of the isolated RNAs was performed. After 12 rounds of selection, the protein-dependent binding ability of the pool increased from 3 to 26%, whereas the filter binding was only 0.2%.

Forty clones were isolated from the twelfth round of selection and were sequenced (Figure 1). Thirty-six of these clones were classified into five families (F1−F5), and the levels of sequence similarity among the families are relatively low. As shown by the red-colored sequences in Figure 1, all of the clones have sequences similar to the region of residues 1484−1506, which includes the *E. coli* 16S rRNA cleavage site. Among these families, F2 bears the highest resemblance to the 16S rRNA sequence: 16 bases in aptamer F2-1 were identical among 23 bases around the cleavage site in 16S rRNA. The locations of these common bases were concentrated in the single-stranded and internal loop regions of 16S rRNA (see Figure 5c). In general, in *in vitro* selections targeting proteins related to RNA cleavage, the sequences of the aptamers that bind to the proteins have no correlation with those of the natural RNA substrates. For example,

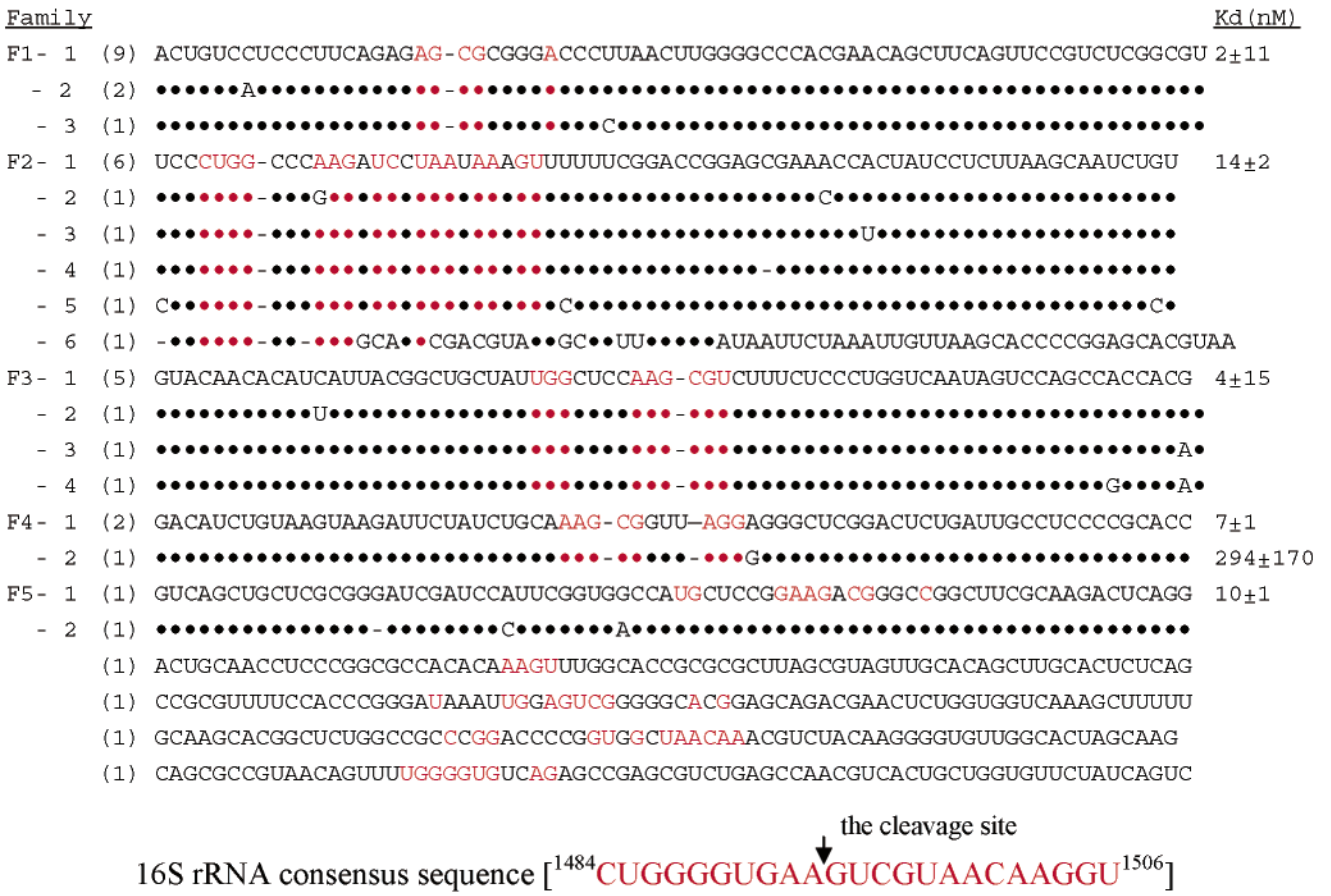

FIGURE 1: Sequences of the random regions of 40 clones selected after 12 rounds of *in vitro* selection. The numbers in parentheses indicate the number of the same clone. Dots represent unchanged nucleotides.

Table 1: Summary of *in Vitro* Selection Experiments

selection and round	[RNA] (nM)	[colicin E3 CRD] (nM)	[tRNA] (nM)	[RNA]:[colicin E3]:[tRNA]	% bound to colicin E3	% bound to filter
N72						
0					3.0	0.1
1	1700	170		50:5:0	4.9	0.2
2	500	150	5000	10:3:100		
3	250	150	5000	5:3:100		
4	150	150	5000	3:3:100		
5	150	150	5000	3:3:100	7.5	0.2
6	150	150		3:3:0		
7	150	150		3:3:0		
8	50	50		1:1:0		
9	50	50		1:1:0	16.8	0.1
10	25	50		0.5:1:0		
11	25	50		0.5:1:0	22.6	0.1
12	50	50		1:1:0	26.1	0.2
D70						
0					3.6	0.03
1	1667	167		10:1:0	5.0	0.04
2	1667	167		10:1:0		
3	1667	167		10:1:0		
4	417	167		2.5:1:0	8.8	0.04
5	417	167		2.5:1:0	21.8	0.03
6	417	167		2.5:1:0		
7	167	167		1:1:0	38.0	0.02
8	167	167		1:1:0	51.5	0.02

aptamers that bind ribosome-inactivating proteins, such as ricin and pepocin, related to the specific digestion of 23S rRNA, have sequences completely different from that of the 23S rRNA cleavage site (*32*, *33*). Thus, to examine the sequence resemblance between the aptamers and 16S rRNA, we characterized the aptamers, especially aptamer F2-1.

Efficiency of Binding of the Aptamers to the Colicin E3 CRD and Inhibition of the Colicin E3 Nuclease Activity. Each aptamer family binds to the colicin E3 CRD with high affinity. The K_d values for binding of the aptamers to the CRD are listed in Figure 1, and each family has K_d values ranging between 2 and 14 nM. The only exception is aptamer F4-2, which has low affinity for the protein, with a K_d of 294 nM. Only one base substitution exists in aptamer F4-2, in comparison to the sequence of aptamer F4-1 ($K_d = 7$ nM). This substitution might have occurred during amplification and cloning after the twelfth round of selection, because such a low-affinity molecule would be difficult to isolate by the selection procedure.

Aptamer F2-1 exhibited specificity for colicin E3, while its affinity for the E4 and E6 colicin families was very low (Figure 2). All of the E3, E4, and E6 colicins cleave the same 16S RNA site, and they are highly related to one another (*40*, *41*). This suggests that aptamer F2-1 may have a sequence that specifically interacts with the colicin E3 CRD, as well as the sequence resembling the 16S rRNA.

Although the sequence of aptamer F2-1 strongly resembles that of the 16S rRNA, the aptamer itself was not cleaved by a treatment with the colicin E3 CRD (0.1 mM) for 1 h at 37 °C (data not shown); instead, the aptamer inhibited the CRD nuclease activity. The inhibition by the aptamer was examined using an *in vitro* translation system with the CRD. The CRD was mixed with the aptamers of each family and incubated at 37 °C for 15 min. Then, the mixture was incubated with *E. coli* ribosomes at 37 °C for 5 min. Using this solution containing the colicin-treated ribosomes and an

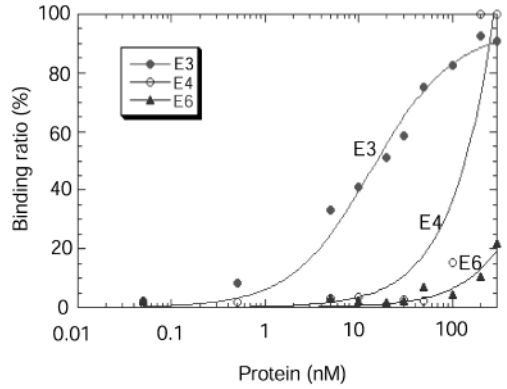

FIGURE 2: Binding curves for binding of aptamer F2-1 to colicins E3, E4, and E6. Different concentrations of the protein were mixed with a fixed concentration (0.6 nM) of aptamer at 37 °C for 1 h, and the percent binding was determined using a filter binding assay.

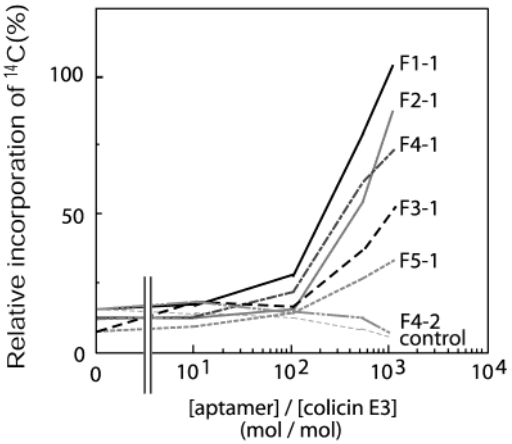

FIGURE 3: Inhibition of the colicin E3 activity by the aptamers in poly(U)-dependent poly(Phe) synthesis. As a control, a randomly selected RNA fragment from the initial pool was used, with a K_d of 147 nM.

S100 fraction extracted from *E. coli*, poly(U)-dependent poly(Phe) synthesis was carried out at 37 °C for 15 min (Figure 3). The colicin-treated ribosomes without the aptamers were deactivated; the translation efficiency was reduced to less than 25% of that without the colicin treatments. By the addition of the aptamers, the efficiency of the poly(Phe) synthesis was restored, although the efficiency depended on the family. The addition of aptamer F1-1 in a 1000-fold excess over the colicin E3 CRD completely restored the protein synthesis, and aptamer F2-1 was also effective. Aptamer F4-2, which has low affinity for colicin E3, did not restore the protein synthesis. Thus, by binding to the CRD, the aptamers inhibit its nuclease activity, and therefore are functional analogues of the colicin E3 immunity protein. In the presence of the immunity protein (0.2 μM), the binding of aptamer F2-1 (0.2 μM) to the colicin E3 CRD (1 μM) was completely inhibited, suggesting that the immunity protein and the aptamers competitively inhibit the colicin E3 nuclease activity.

Second in Vitro Selection, Using a Doped Pool Derived from Aptamer F2-1, and Secondary Structure Prediction. To determine the secondary structure of aptamer F2-1 and to identify the conserved bases in the aptamer, we performed a second *in vitro* selection using a doped sequence population of aptamer F2-1, the sequence of which is most similar to that of 16S rRNA among the aptamers. Each position in the 70-mer sequence of aptamer F2-1, corresponding to the original random region, contained 50% wild-type residues and 17% of each non-wild-type residue. We employed a relatively high level of randomization for the pool to obtain a wider variety of aptamer sequences and to determine their diversity (*42*).

The doped selection provided information about the conserved bases in aptamer F2-1 and its secondary structure. After eight rounds of selection and amplification, the ability of the pool to bind the protein increased to 52% (D70 in Table 1). In the pool from the eighth round, 77 clones were isolated and sequenced (Figures 4 and 5 and Supporting Information). Thirteen RNAs, which were randomly selected from the clones, had K_d's of 1−42 nM. Although the selection began with the highly randomized pool, 76 sequences among the 77 clones resembled the sequence of aptamer F2-1, with sequences similar to AAGUCGUAACAAGG between residue 1492 and 1505 in 16S rRNA. In addition, a high level of covariations that maintain the Watson−Crick and G·U base pairs was found in the 76 aptamers. These covariations enabled the prediction of the secondary structure of aptamer F2-1. The predicted aptamer F2-1 secondary structure, which contains the three complementary regions of stems 1−3, is shown in Figure 4. The sequences related to AAGUCGUAACAAGG are located in the regions of stem 2 and loop 2. This structure was completely different from that predicted by the program MulFold (*43*), which contains a large hairpin structure at positions 9−58, including a loop region from position 25 to 44. The structure shown in Figure 4 was also confirmed by enzymatic and chemical probing. Aptamer F2-1 was digested mainly at two regions, residues 49−51 and 81−83, by nuclease S1, while it was significantly modified at residues A43, A45, A46, A69, and A72 by DEP and U44, U74, U81, and U82 by CMCT (see lane 4 of Figure 5). Single-stranded regions of RNA structures are sensitive to S1 digestion and chemical modifications (*39*).

The conserved bases among these aptamers are concentrated around the internal loop 1, suggesting that the surrounding region, including stems 1−3, is also important for the binding to the protein. This was confirmed by the binding assay of a series of aptamer F2-1 deletion mutants. The affinities of the mutants, which lacked either stem 1′ (fragment 1−81; $K_d = 178 \pm 69$ nM) or stem−loop 3 and stem 1′ (fragment 1−58; $K_d = 175 \pm 30$ nM), for the protein decreased 10-fold, in comparison to that of aptamer F2-1 ($K_d = 14 \pm 2$ nM). Thus, for strong binding to the protein, the tertiary structure comprising the entire length of the aptamer is required. A CMCT modification interference experiment also showed that a large area, including loop 1, stem 2′, loop 2, and loop 3, of the aptamer interacts with the colicin E3 CRD (Figure 5). The 5′-labeled aptamer F2-1 and its complex with the colicin E3 CRD were treated with CMCT, and the modification sites were analyzed by gel electrophoresis of the DNA products obtained with AMV reverse transcriptase. The CMCT modifications at U49−U54, U74, U81, and U82 of aptamer F2-1 were blocked by the binding of the protein (Figure 5, lanes 4 and 5). Since U51−U54 are in stem 2′, the CMCT modification of this region was very weak without the protein. However, formation of the aptamer−protein complex completely interfered with the modification.

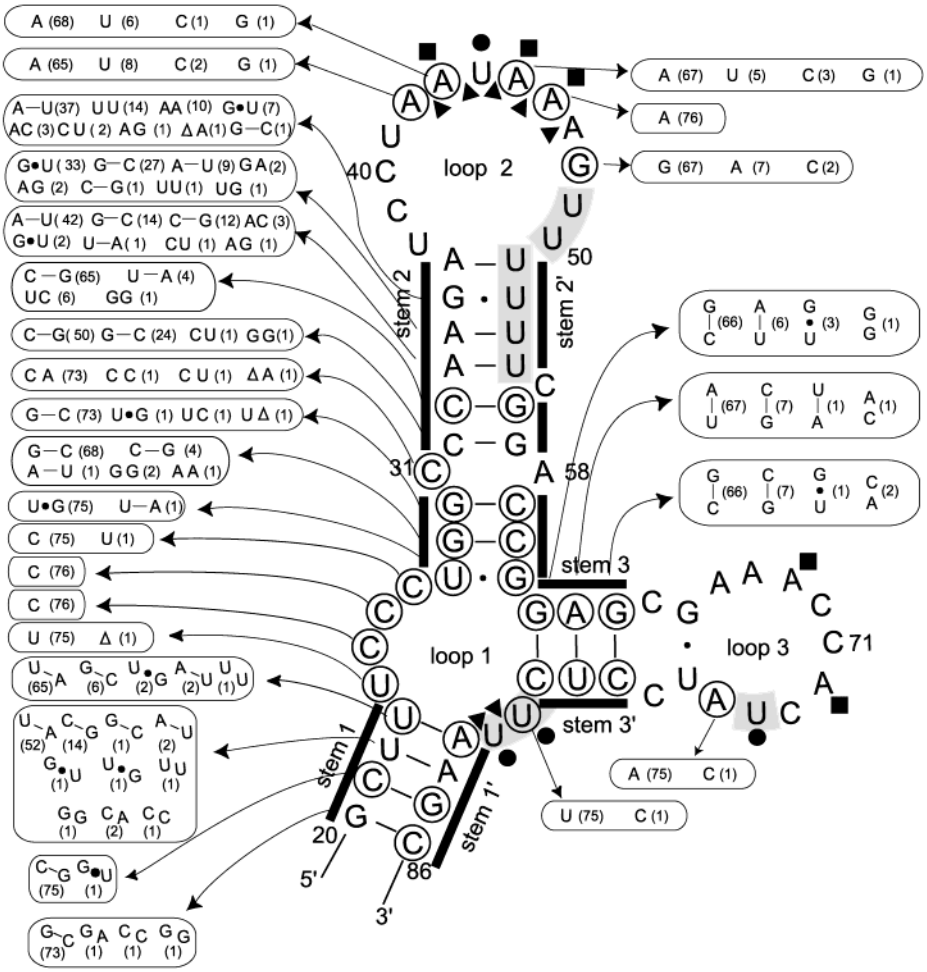

FIGURE 4: Predicted secondary structure of aptamer F2-1. The secondary structure of aptamer F2-1 was predicted from covariations observed in the sequences obtained in a doped reselection (see the Supporting Information). Bold lines represent putative base-paired nucleotides, as supported by covariations. Bases conserved in at least 65 of 76 clones that were sequenced are encircled. Major S1 nuclease cleavage sites are represented with black triangles. The nucleotides that were significantly modified by DEP and CMCT are represented with black squares and black circles, respectively. Regions where formation of the aptamer−protein complex interfered with CMCT modification are shaded.

Structural Comparison between the F2-1 Aptamer and the 16S rRNA Cleavage Region. We compared the secondary structures of aptamer F2-1 and 16S rRNA. The ^{27}CUGGCCCAAGAUCCUAAUAAAGU^{49} sequence in aptamer F2-1 is similar to the ^{1484}CUGGGGUGAAGUCGUAACAAGGU^{1506} sequence, including the cleavage site between A1493 and G1494, in 16S rRNA. This cleavage site is involved in the decoding region adjacent to a stem−loop structure from residue 1409 to 1491 in 16S rRNA. We associated the secondary structure of aptamer F2-1 with that of the region around the 16S rRNA cleavage site, and rearranged the model of the secondary structure of the aptamer, as shown in Figure 6b.

In Figure 6, the regions in aptamer F2-1 (stem−loop 2 in Figure 6b) and 16S rRNA (C1404−G1416 and C1484−U1506 in Figure 6c) with structural similarities are highlighted in yellow. Two nucleotides, U50 and U51, in aptamer F2-1 could be regarded as a connection between U1506 and a nucleotide around C1404−U1406 of *E. coli* 16S rRNA. The crystal structure of this 16S rRNA region from *Thermus thermophilus* suggests the possibility that U1506 is located near C1404 (Figure 6d) (*44*). Although the *T. thermophilus* 16S rRNA is not the natural target of colicin E3, the 30S ribosomal subunit of *T. thermophilus* binds the E517Q mutant of the CRD, which has an affinity for *E. coli* ribosomes, but does not cleave 16S rRNA (*25*). The remarkable difference in the highlighted regions is the structure of the cleavage sites. In 16S rRNA, this region is part of the decoding region and forms a flexible structure with non-Watson−Crick base pairs, but the corresponding region in aptamer F2-1 forms a duplex structure with Watson−Crick and G·U base pairs. This structural difference between aptamer F2-1 and the decoding region can explain why the aptamer is not cleaved by colicin E3, and suggests that the duplex structure of the aptamer can resist the colicin E3 nuclease activity. This implies that the disruption of the base pairs in this region would cause aptamer F2-1 to become sensitive to the colicin E3 cleavage. Thus, we prepared a variant in which the sequence between A37 and U53 of aptamer F2-1 was replaced with a UCGUAACAAGGCCG sequence, corresponding to ^{1495}UCGUAACAAGG^{1505} and ^{1403}CCG^{1405} of 16S rRNA, and examined its colicin E3 sensitivity. After the 5′-^{32}P-labeled variant (120 nM) was treated with the colicin E3 CRD (90 nM) at 30 °C for 24 h, the variant was cleaved at a position corresponding to C1407 of 16S rRNA or C52 of the variant aptamer, but the original F2-1 aptamer was not sensitive at this position under the same conditions (data not shown). This cleavage site was

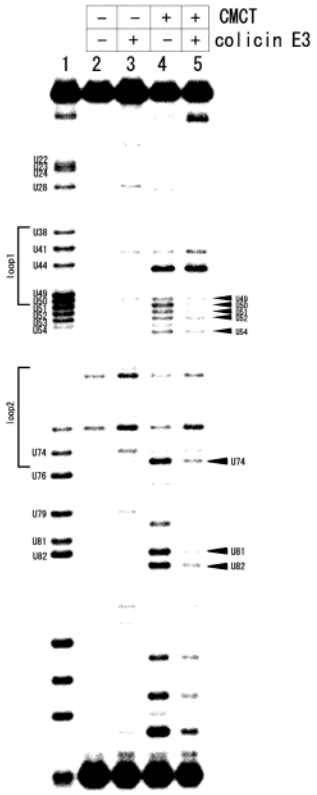

FIGURE 5: CMCT modification interference of the binding of aptamer F2-1 to colicin E3. CMCT modifications were detected by reverse transcription of aptamer F2-1. The cDNA products obtained by primer extension revealed the modified nucleotides. Incubations were carried out in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of colicin E3. Lanes 2 and 3 are incubation controls in the absence of CMCT. Positions with altered reactivities are denoted with triangles. As a reference, lane 1 shows a reverse transcription reaction of aptamer F2-1 with ddATP.

on the opposite side of A1493, and the cleavage was not efficient. However, the variant of aptamer F2-1, which has a sequence similar to that of the decoding region of 16S rRNA, became sensitive to the colicin E3 cleavage.

Neither the sequence nor the structure of stem−loop 3 in F2-1 bear any clear resemblance to those of 16S rRNA. However, for tight binding to the CRD, the entire length of the aptamer is required. A recent *in silico* analysis of the interaction between colicin E3 and 16S rRNA predicted several sites, such as U955, A964, and G1198, that could interact with colicin E3 (*25*, *26*). This suggests that some residues in stem−loop 3 also interact with the protein and specifically recognize colicin E3.

From the resemblance, colicin E3 may interact with the regions including residues 1404−1416 and 1484−1506 and other residues in 16S rRNA. This prediction agrees with the results obtained from the model for docking of colicin E3 to the 30S ribosomal subunit from *T. thermophilus* (*25*, *26*). In this docking model, Lys544 of colicin E3 interacts with G1405 of 16S rRNA (the nucleotide numbering corresponds to the *E. coli* numbering), while other interactions include Asn490 with C1409, Lys549 and Tyr550 with A1492, and Arg495 with A1493 (*26*).

Conclusions. Anti-colicin E3 aptamers were isolated by *in vitro* selection, using the colicin E3 CRD. These aptamers specifically bound to colicin E3 and inhibited the nuclease activity. The secondary structure of aptamer F2-1 was predicted from the covariations obtained by the doped aptamer selection. Aptamer F2-1 is similar in sequence and structure to the region around the cleavage site in 16S rRNA, suggesting that the aptamer mimics the local structure of 16S rRNA. Thus, aptamer F2-1 could be used for the structural analysis of the interaction with colicin E3, in place of 16S rRNA. Furthermore, the aptamers work as analogues of the colicin E3 immunity protein, and the expression of the aptamers in a colicin-sensitive cell could confer resistance to colicin E3. This implies that the aptamers could be used as selection markers, such as anti-aminoglycoside aptamers, for the isolation of certain *E. coli* mutants (*45*).

SUPPORTING INFORMATION AVAILABLE

A table of the 77 clone sequences obtained with the doped selection. This material is available free of charge via the Internet at http://pubs.acs.org.

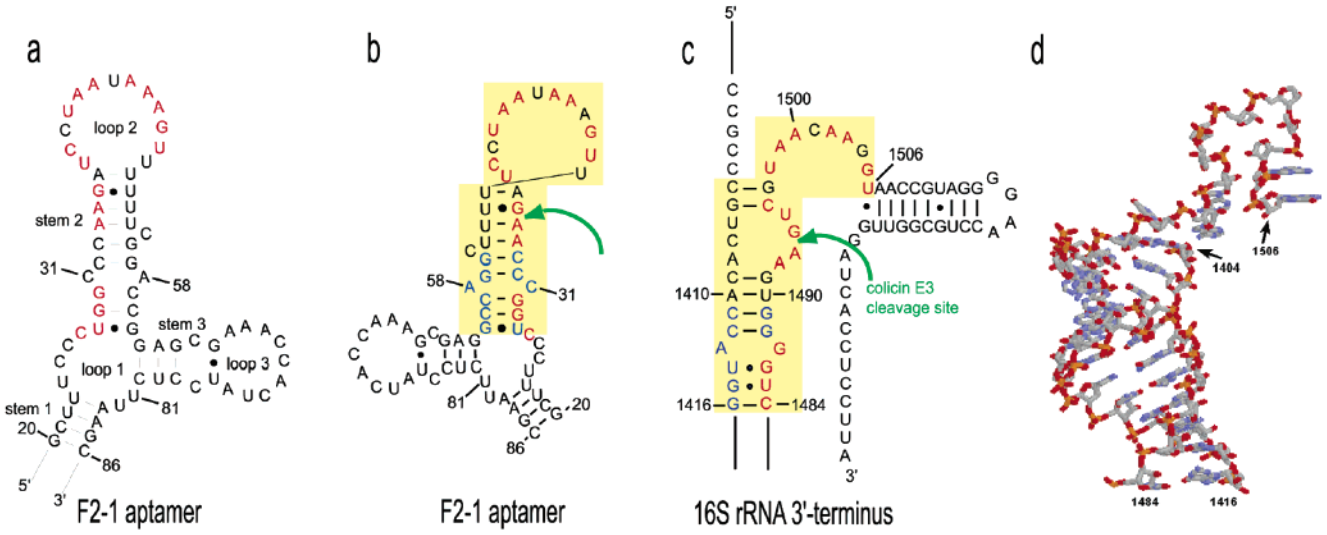

FIGURE 6: Predicted secondary structure of aptamer F2-1 and partial secondary and tertiary structures of 16S rRNA. Predicted secondary structure of aptamer F2-1 (a) and its rearranged model (b). Partial secondary structure of *E. coli* 16S rRNA (c) and crystal structure of the aptamer-related region of the 16S rRNA portion in the *T. thermophilus* 30S ribosomal subunits (d) (*38*). The yellow-colored regions represent the structural similarity between aptamer F2-1 and 16S rRNA. The corresponding *E. coli* numbering is used for the *T. thermophilus* 16S rRNA crystal structure.

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