

Aptamers That Preferentially Bind Type IVB Pili and Inhibit Human Monocytic-Cell Invasion by *Salmonella enterica* Serovar Typhi

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Salmonella enterica serovar Typhi is an important pathogen exclusively for humans and causes typhoid or enteric fever. It has been shown that type IVB pili, encoded by the *S. enterica* serovar Typhi *pil* operon located in *Salmonella* pathogenicity island 7, are important in the pathogenic process. In this study, by using both an adhesion-invasion assay and fluorescence quantitative PCR analysis, we demonstrated that the entry of type IVB piliated *S. enterica* serovar Typhi A21-6 (*pil*⁺ Km^r) into human THP-1 monocytic cells was greater than that of a nonpiliated *S. enterica* serovar Typhi *pilS*::Km^r (*pil* mutant) strain. We have applied a systematic evolution of ligands by exponential enrichment approach to select oligonucleotides (aptamers) as ligands that specifically bind to type IVB pili. Using this approach, we identified a high-affinity single-stranded RNA aptamer (S-PS_{8.4}) as a type IVB pilus-specific ligand and further found that the selected aptamer (S-PS_{8.4}) could significantly inhibit the entry of the piliated strain (but not that of the nonpiliated strain) into human THP-1 cells. The binding affinities between aptamers and pre-PilS (structural protein of type IVB pili) were determined by nitrocellulose filter-binding assays, and the K_d value was determined to be 8.56 nM for the S-PS_{8.4} aptamer alone. As an example of an aptamer against type IVB pili of *S. enterica* serovar Typhi, the aptamer S-PS_{8.4} can serve as a tool for analysis of bacterial type IVB pilus-host cell interactions and may yield information for the development of putative new drugs against *S. enterica* serovar Typhi bacterial infections, useful both in prevention of infection and in therapeutic treatment.

Of the more than 2,300 closely related *Salmonella* serovars identified, *Salmonella enterica* serovar Typhi is an important pathogen exclusively for humans and can be transmitted through contaminated food and water. It causes typhoid or enteric fever, which is a serious public health problem in developing countries. The genome of *S. enterica* serovar Typhi contains three large inserts (pathogenicity islands) (11), relative to the chromosome of *Salmonella enterica* serovar Typhimurium, which is normally noninvasive for humans. The type IVB *pil* operon of *S. enterica* serovar Typhi is located in *Salmonella* pathogenicity island 7 (18) and contains a *pilS* gene encoding the structural pilin (36, 37). It has been demonstrated that a *pilS* mutant of *S. enterica* serovar Typhi exhibited much-reduced adhesion to and invasion of human epithelial gastrointestinal cells in vitro and that purified soluble pre-PilS protein, retaining the signal sequence normally cleaved when the protein is excreted to form insoluble pili based on polymerized PilS, inhibited bacterial invasion (37). The structure of the N-terminally truncated type IVB structural pilin from *S. enterica* serovar Typhi was determined by nuclear magnetic resonance analysis (34). Type IVB pili, composed largely of polymerized PilS protein, also mediate bacterial self-association, but only when the presumptive minor pilus proteins PilV1 and

PilV2 are not expressed (15). Bacterial self-association is an important virulence trait in enterotoxigenic strains of *Escherichia coli* and in *Vibrio cholerae* (1). These data indicated that the structural protein PilS of the type IVB pili might play important roles in the pathogenesis of *S. enterica* serovar Typhi in humans.

The SELEX (systematic evolution of ligands by exponential enrichment) method (28) is an oligonucleotide-based combinatorial library selection procedure that has been used extensively to isolate ligands (aptamers) that bind to proteins (3, 6, 9, 22, 32), cell surface epitopes (19, 21), and other targets (4, 12, 13, 17). Although in recent years SELEX has become increasingly important in the study of functions of proteins, as well as in the fields of drug discovery and identification of antagonists against many functional proteins, this in vitro selection strategy to generate inhibitors of the functions of bacterial proteins remains underutilized. Aptamers have several potential advantages over antibodies and antibiotics. Aptamers have high affinity and specificity for their targets and can be considered oligonucleotide analogs of antibodies. Being smaller than antibodies, aptamers are better candidates for cell penetration and blood clearance. A variety of chemical modifications, such as fluorescent probes, cross-linking reagents, and modifications to the backbone or specific bases by fluorine (2'-hydroxyl groups of the ribose moieties are replaced with fluorine) (7), can be introduced, thereby adding stability and functionality. Moreover, aptamers are nonimmunogenic and

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therefore do not cause side effects resulting from unwanted immune responses in hosts.

Both single-stranded DNA (ssDNA) and single-stranded RNA (ssRNA) are candidates for aptamers. Comparing ssRNA with ssDNA, ssRNA might have a more variable dimensional structure than ssDNA. As for the oligonucleotide structure, G-C, A-U, and G-U pairings can occur in ssRNA but only G-C and A-U pairings occur in ssDNA. So we chose ssRNA aptamers which had a richer spatial configuration and facilitated interaction with the PilS protein.

During natural *Salmonella* bacterial infections, monocytes/macrophages serve as key effector cells of the immune response. In order to further investigate the pathogenic roles of the PilS protein of the type IVB pili, we applied a SELEX approach to identify oligonucleotides (aptamers) as ligands that bind to type IVB pili and then investigated the effects of the aptamers on *S. enterica* serovar Typhi adhesion to and invasion of the human monocytic leukemia cell line THP-1. We have identified a single ssRNA aptamer (S-PS_{8,4}) as a ligand of the *S. enterica* serovar Typhi type IVB pili and found it significantly inhibited the entry of the piliated strain (but not that of the nonpiliated strain) into human THP-1 cells.

MATERIALS AND METHODS

Materials. *E. coli* DH5 α and BL21(DE3)/plysS were used as described previously (37). *S. enterica* serovar Typhi A21-6 (*pil*⁺) (37) was generated by inserting a *tac* promoter between *pilM* and *pilN* with the 575-nucleotide *pilM-pilN* intergenic sequence removed. Therefore, transcription of the *pilN* through *pilV* genes of the *pil* operon was under the control of the *tac* promoter. This construct does not disrupt other genes, such as that for lipopolysaccharide, that are controlled by different promoters. The nonpiliated strain *S. enterica* serovar Typhi *pilS*::Km^r (37) was generated by inserting a kanamycin resistance gene into the *pilS* gene so that the destruction of the pilin operon did not influence other gene expression that was under the control of different promoters. All bacterial strains were grown in Luria-Bertani broth (LB) as previously described for 14 to 16 h at 30°C (37). Solid medium contained 1.5% (wt/vol) agar. Human acute monocytic leukemia cell line THP-1 cells were cultured in RPMI 1640 medium (Gibco) with 10% (vol/vol) fetal bovine serum (Gibco).

Expression and purification of pre-PilS–glutathione S-transferase (GST) fusion protein. The PCR product of the *pilS* gene (which encodes the pre-PilS protein, in which the mature PilS protein is preceded by a signal sequence) was digested with EcoRI and BamHI, purified, and inserted into the EcoRI and BamHI sites of plasmid pGEX-2T to yield plasmid pGEXS5, in which *pilS* was fused in frame with the gene for GST. pGEXS5 was transformed into *E. coli* BL21(DE3)/plysS. After growth to logarithmic phase, the strain hosting the plasmid encoding the pre-PilS–GST fusion protein was induced with 1 mM IPTG for 4 h. Cells were sonicated in phosphate-buffered saline (PBS) and the GST-tagged protein purified through glutathione-Sepharose 4B (Amersham Biosciences).

Western blot analysis. The purified pre-PilS–GST fusion protein and GST, as a control, were separated by 12% (wt/vol) sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Fat-free milk powder solution (10%, wt/vol) was used for blocking. The membrane was incubated with a primary anti-pre-PilS–GST polyclonal antibody (37) for 1 h at room temperature, washed three times, and then incubated with a 1:1,000 dilution of alkaline phosphatase-conjugated anti-mouse immunoglobulin G. Color was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

SELEX. SELEX procedures were generally performed as previously described (31, 33, 38). The oligonucleotide template was synthesized as a single-stranded 88-mer with the sequence 5'-GCGGAATTCTAATACGACTACTATAGGG AACAGTCCGAGCC-N₃₀-GGGTCAATGCGTCATA-3', where the central N₃₀ represents random oligonucleotides based on equal incorporation of A, G, C, and T at each position. The complementary strand was synthesized using the DNA polymerase I Klenow fragment with primer 2 (5'-GCGGGATCCTATGACGATTGACCC-3'). The dsDNA molecules were generated by PCR amplification using primer 2 and primer 1 (5'-GCGGAATTCTAATACGACTACT

ATAGGGAACAGTCCGAGCC-3'), which contains the T7 polymerase promoter (underlined). The initial RNA random library was prepared by incubation with T7 RNA polymerase for 2 h at 37°C in a 100- μ l reaction mixture (2 to 5 μ g DNA, 0.5 mM UTP, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 20 μ l 5 \times transcription buffer [Promega], 10 mM dithiothreitol [DTT], 100 U RNasin, 40 U T7 RNA polymerase), and the transcribed RNAs were purified using an Rnaid Kit (Qbiogene). To initiate *in vitro* selection, purified random RNAs were incubated in binding buffer (25 mM Tris-HCl, 50 mM KCl, 200 mM NaCl, 0.2 mM EDTA, 5% [vol/vol] glycerol, 0.5 mM DTT) for 15 min at 20 to 25°C, with GST–pre-PilS protein bound to glutathione-Sepharose 4B. Following washing with at least 40 column volumes of binding buffer, bound RNAs were eluted with 1 M NaCl in binding buffer and purified using the Rnaid Kit. After reverse transcription (RT)-PCR amplification with primer 1 and primer 2, the resulting DNA was transcribed with T7 RNA polymerase for 2 h at 37°C in a 100- μ l reaction mixture (2 μ g to 5 μ g DNA, 0.5 mM UTP, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 20 μ l 5 \times transcription buffer [Promega], 10 mM DTT, 100 U RNasin, 40 U T7 RNA polymerase) and this RNA pool was used in the next round of selection. From round three to round eight, RNA pools were first bound to GST-Sepharose 4B to remove nonspecifically bound RNA and then bound to pre-PilS–GST–Sepharose 4B material.

In vitro transcription. The RNA products of the test application and of a control in which the T7 RNA polymerase was omitted were quantitated by liquid scintillation counting after 10 min, 30 min, and 60 min of incubation in 20- μ l reaction volumes (1 μ g DNA, 12.5 μ M UTP, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 50 μ Ci [α -³²P]UTP, 4 μ l 5 \times transcription buffer [Promega], 10 mM DTT, 25 U RNasin, 20 U T7 RNA polymerase). Label incorporation into RNA of the test sample increased over time and exceeded that of the control. Label incorporation decreased slightly after 60 min, perhaps due to RNA degradation.

Cloning and sequencing. After eight rounds of selection, RT-PCR products were digested with EcoRI and BamHI and then subcloned into pUC19. The bank was transformed into *E. coli* DH5 α . Plasmid DNA was isolated from individual clones, purified, and analyzed by sequencing.

Adhesion and invasion assay using mixed infections. Human acute monocytic leukemia THP-1 cells were maintained and prepared for bacterial adherence and invasion assays as previously described (23). THP-1 cells were grown in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum to a density of 2×10^5 to 1×10^6 /ml. Fresh cells (1×10^6 /ml) were added to each well of a six-well plate using 1 ml/well. Then 1 ml of a dilution (in RPMI 1640 medium supplemented with 10% [vol/vol] fetal bovine serum) of an equal mixture of two *S. enterica* serovar Typhi strains, either *S. enterica* serovar Typhi *pilS*::Km^r or Typhi A21-6 (*pil*⁺ Km^r) and a wild-type strain of *S. enterica* serovar Typhi J341 (*pil*⁺), was added and mixed with the monocytes at a ratio of 30:1, 10:1, or 1:1 (bacterial cells to eukaryotic cells). Next, the mixtures were incubated at 37°C in a 5% (vol/vol) CO₂ atmosphere for 2 h. Bacteria that did not enter the eukaryotic cells were removed by three washes with PBS or eliminated by incubation with gentamicin (200 μ g/ml) for 1 h, followed by washing twice with PBS. Intracellular bacteria were recovered by eukaryotic cell lysis with 0.01% (wt/vol) Triton X-100 for 20 min and enumerated on both LB agar plates and LB agar plates supplemented with kanamycin (both the *S. enterica* serovar Typhi A21-6 wild-type strain and the *S. enterica* serovar Typhi *pilS* mutant were Km^r). Because mixtures of the wild-type and mutant strains *S. enterica* serovar Typhi A21-6 (*pil*⁺ Km^r) and *S. enterica* serovar Typhi *pilS*::Km^r were used, the procedure decreased the variability between independent trials.

Inhibition of cell invasion and adherence by aptamers. THP-1 cells were grown in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum to a density of 2×10^5 to 1×10^6 /ml. *S. enterica* serovar Typhi strains (*S. enterica* serovar Typhi A21-6 and *S. enterica* serovar Typhi *pilS*::Km^r) were grown to stationary culture in LB for 14 to 16 h at 30°C to reach an optical density at 600 nm of 0.5 to 0.7. Prior to infection, the bacteria were incubated with 2 μ g RNA aptamer S-PS_{8,4} in 100 μ l diethyl pyrocarbonate-treated PBS at 20 to 25°C for 15 min. The treated bacteria were added to THP-1 cells (2.5×10^5 cells/well) in 24-well plates at a ratio of 12:1 (bacterial cells to eukaryotic cells). Following incubation at 37°C for 2 h in a 5% (vol/vol) CO₂ atmosphere, cells were harvested by centrifugation at $900 \times g$ for 10 min and washed thrice with PBS. Gentamicin (200 μ g/ml) was added to kill residual extracellular bacteria. After further incubation for 1 h, the THP-1 cells were washed three more times and lysed with 0.01% (wt/vol) Triton X-100 to release intracellular bacteria. The bacteria were enumerated on LB agar plates with kanamycin. Basic controls for each experiment included bacteria only (no THP-1 cells) and THP-1 cells only. The percentage of bacterial adhesion and invasion inhibition afforded by the aptamer was determined as follows: percent inhibition = [(bacterial number with no S-PS_{8,4} – bacterial number with S-PS_{8,4})/bacterial number with no S-PS_{8,4}] \times 100%.

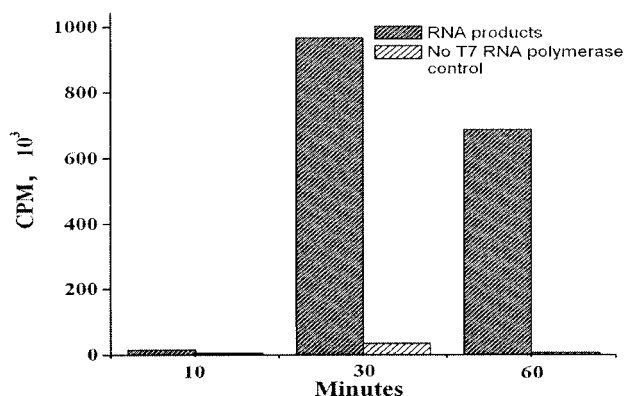


FIG. 1. In vitro transcription in the SELEX procedure. Labeled RNA products were quantitated by liquid scintillation counting at 10 min, 30 min, and 60 min after initiation of transcription, respectively. Label incorporation into RNA products was increasingly higher than that of the control (no T7 RNA polymerase). The label incorporated into RNA product was significantly increased after 30 min, while label incorporation dropped slightly after 60 min, perhaps due to RNA degradation.

Real-time fluorescence quantitative PCR (FQ-PCR) assay. Real-time FQ-PCR templates were prepared as follows. Experiments involving interaction of bacteria with THP-1 cells were performed basically as described above, except that cells were lysed with 0.1 M Tris-HCl (pH 8.0), 0.01 M EDTA, 1% (wt/vol) SDS, 0.04 M DTT, and 0.2 g/liter proteinase K at 55°C for 1 h after gentamicin treatment. Total DNA was purified from the cells, extracted with phenol-chloroform, and precipitated with 2 volumes of 100% ethanol. Quantification of intracellular bacteria was achieved using real-time quantitative PCR with the QuantiTect SYBR Green PCR Handbook Kit (QIAGEN). The DNA templates for real-time quantitative PCR in each sample were equal (35 ng). In this technique, the *pilS* gene was specifically amplified from *S. enterica* serovar Typhi A21-6 with primers P^{F1} (5'-AATGGCTCGAGGAACTGTGAAGCGA3') and P^R (5'-TTCATCATGCCAATGGTGTTCGTGGC3') or from *S. enterica* serovar Typhi *pilS*::Km^r with primers P^{F2} (5'-TCGATAGATTGTCGCACCTGATTGC C3') and P^R, respectively. Forward primer P^{F2}, for amplification of *pilS*::Km^r mutant DNA, lies in the Km^r gene and contains part of the kanamycin resistance gene sequence. The primers were designed to yield PCR products of similar sizes (each ca. 350 bp), and tests using mixtures of equal numbers of the two bacterial strains confirmed that the primer pairs were equally efficient in amplification (data not shown). The standard curves were generated as described previously (35). To determine the effects of aptamer S-PS_{8,4} on *S. enterica* serovar Typhi infection, the percentage of inhibition of bacterial intracellularization afforded by the aptamer was calculated as follows: percent inhibition = [(*pilS* gene concentration with no S-PS_{8,4} - *pilS* gene concentration with S-PS_{8,4})/*pilS* gene concentration with no S-PS_{8,4}] × 100%.

Nitrocellulose filter-binding assay. ³²P-labeled RNA was produced by in vitro transcription with ³²P-labeled UTP, nucleoside triphosphates (GTP, CTP, UTP, and ATP), and T7 RNA polymerase. Each 20- μ l reaction mixture containing 25 mM Tris-HCl (pH 7.5), 50 mM KCl, 200 mM NaCl, 0.2 mM EDTA, 5% (vol/vol) glycerol, 0.5 mM DTT, and 10 pmol of RNA was allowed to equilibrate with variable (excess) concentrations of protein at 20 to 25°C for 15 min. Samples were filtered through nitrocellulose disks prewetted with 25 mM Tris-HCl (pH 7.5) and immediately rinsed with 3 ml of the same solution. The disks were dried and counted by scintillation counting. Dissociation constants were determined as previously described (10, 24). The data points of binding curves were fitted to the equation $F = P/(K_d + P)$ to determine the dissociation constants by Origin 6.0 (Microcal Software Inc.), where F is the fraction of RNA bound, P is the concentration of protein, and K_d is the dissociation constant.

RESULTS

Isolation of RNA aptamers binding to type IVB pili of *S. enterica* serovar Typhi. Before the SELEX selection experiment (below), the random RNA pools obtained at various times after initiation of transcription were quantitated by liq-

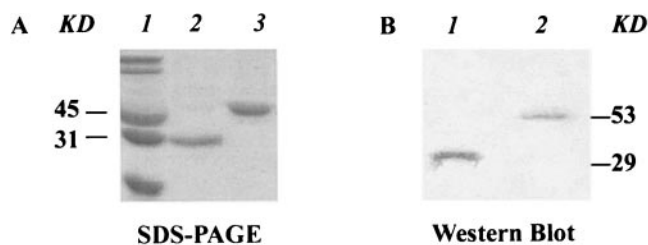


FIG. 2. Expression and purification of pre-PilS-GST fusion protein. (A) SDS-polyacrylamide gel electrophoresis with Coomassie blue R-250 staining. Lane 1, molecular mass standard. Lane 2, GST protein (29 kDa). Lane 3, pre-PilS-GST fusion protein (53 kDa). (B) Western blot assay with anti-pre-PilS-GST antibody. Lane 1, GST protein (29 kDa). Lane 2, pre-PilS-GST fusion protein (53 kDa). KD, kilodaltons.

uid scintillation counting (Fig. 1). After 10 min, label incorporation into transcription products was detectable. The level of RNA product was significantly increased after 30 min, while label incorporation dropped slightly after 60 min, perhaps due to RNA degradation.

To isolate aptamers that specifically bind to type IVB pili of *S. enterica* serovar Typhi, we utilized the pilin structural protein, tagged with GST, as a selection target. The pre-PilS-GST fusion protein was expressed in and purified from *E. coli* (Fig. 2). After synthesis of the complementary strands from the ssDNA library, the dsDNA library was amplified by PCR. Then the dsDNAs were employed as templates for in vitro transcription. An RNA pool containing randomized 30-nucleotide inserts ($>10^{12}$ molecules) was synthesized. The synthesized random RNA pool was added to pre-PilS-GST-Sepharose 4B beads to select aptamers which might bind to the pre-PilS protein. To ensure the isolation of aptamers of high affinity and specificity, preselections were used, from the third round, to remove RNAs binding to GST-Sepharose 4B. Eight rounds of selection were performed. The final (eighth) RNA pool was reverse transcribed by RT-PCR and subcloned into pUC19 (EcoRI and BamHI) prior to analysis by sequencing. The size of the RT-PCR product was 98 bp, which was the same as the size of the dsDNA (Fig. 3).

Aptamer S-PS_{8,4} secondary-structure prediction. After eight rounds of selection, 14 individual clones that bound to the pre-PilS-GST-Sepharose 4B beads were selected and se-

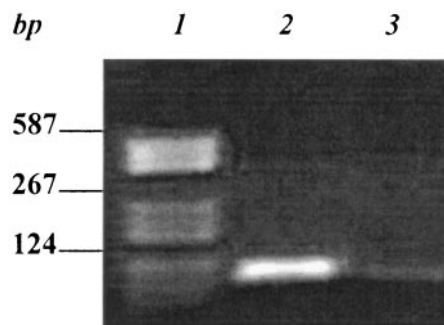


FIG. 3. Products from the eighth and final round of RT-PCR in the SELEX procedure. Lane 1, DNA molecular weight marker. Lane 2, eighth-round RT-PCR products (98 bp). Lane 3, negative control (no reverse transcriptase).

TABLE 1. Aptamers selected by the SELEX procedure

Aptamer	Frequency	RNA aptamer sequence (N ₃₀) ^a			
S-PS _{8,4}	5/14	UCACUG	UUAUCCGAUAGC	<u>A G C G C GG</u>	G A U G A
S-PS _{8,3}	2/14	A	UUA CC UA G	<u>A G C G</u>	GGAUAAA GUA UAG G U U
S-PS _{8,2}	1/14	CA	U CG A GG	<u>A GG C G</u>	GGAUUUUCG A U G AGU U
S-PS _{8,5}	1/14	G A G U A C	G	<u>A G C G</u>	GGUAU GUAUCG GGUGAU
S-PS _{8,6}	1/14	GAAGGGGU	CC G	<u>GCUC G</u>	GG UGAG G G UCG GGU
S-PS _{8,9}	1/14	A	GCU AG CG G G	<u>GGGG CUCG AC GG</u>	U G G U G GGU U
S-PS _{8,7}	1/14		U AG CG G G	<u>A G CUUGGACCUGG</u>	U G G UCGCGGC
S-PS _{8,1}	1/14	UUGG GAU	C A GCUCG	<u>G C G C UGG</u>	A G G A GG GGC
S-PS _{8,8}	1/14	AACUG U A	C AUG	<u>G G C G C</u>	C AA CA G G G AGU UAGC

^a Consensus regions within the 14 individual aptamers are underlined.

quenced. Among them, nine clones were unique and all 14 individual RNAs were aligned using the Clustal software package (Table 1). A consensus sequence (underlined and present eight times in the 14 clones) within individual RNA aptamers was AGCG-(X)-GG. The six RNA aptamers that had no consensus regions (Table 1) perhaps belonged to other small aptamer families. It is interesting that clone S-PS_{8,4} appeared five times in the RNA aptamer pool. The secondary structure of the S-PS_{8,4} RNA aptamer was predicted with the RNA structure program (Fig. 4) (version 4.2; D. H. Mathews, University of Rochester Medical Center [http://www.rna.urmc.Rochester.edu/RNAstructure.zip]). The predicted secondary structure of S-PS_{8,4} shows that the consensus AGCG-(X)-GG sequence occurs in the terminal loop of a stem-loop structure, suggesting that this stem-loop might be the site of binding to the target protein. There are two possible secondary structures (Fig. 4A and B) for the S-PS_{8,4} RNA aptamer. These two predicted secondary structures are equally likely from the sequence analysis of aptamer S-PS_{8,4}, and the free energies of the two predicted secondary structures are very similar. The only difference between the two predicted structures lies in their top stem-loops. In Fig. 4B, 14 nucleotides are connected to form the top stem-loop, but in Fig. 4A, the same 14 nucleotides form the two stem-loops, 5 nucleotides connecting to form the small top loop and the other 9 nucleotides forming the larger one.

A pool of RNA aptamers significantly inhibited *S. enterica* serovar Typhi invasion of THP-1 cells. Based on previous reports, an obvious improvement in aptamer-protein binding was usually apparent after five rounds of amplification (6, 12). In this study, we decided to examine the biological activities of aptamers after the fifth, seventh, and eighth rounds of selection. Compared with a control containing no RNA, addition of the RNA aptamer pools caused significant inhibition of bacterial infection in the real-time FQ-PCR assay (Fig. 5A and B). The inhibitory effects of the RNA aptamer pool increased with advancing cycles (Fig. 5A and B). Moreover, the inhibitory effect of the eighth-round aptamer pool was concentration dependent (Fig. 6). With the addition of 6.1 μ g of the RNA aptamer pool from the fifth, seventh, or eighth amplification round, cell invasion by *S. enterica* serovar Typhi A21-6 decreased to 66%, 3%, and 0.3%, respectively, compared to the cell invasion level seen in the RNA-free control (Fig. 5B). The melting curves of the PCR products suggested that there were no nonspecific PCR products in the PCRs (Fig. 5C). After the eighth round of selection, high-affinity RNA aptamers were

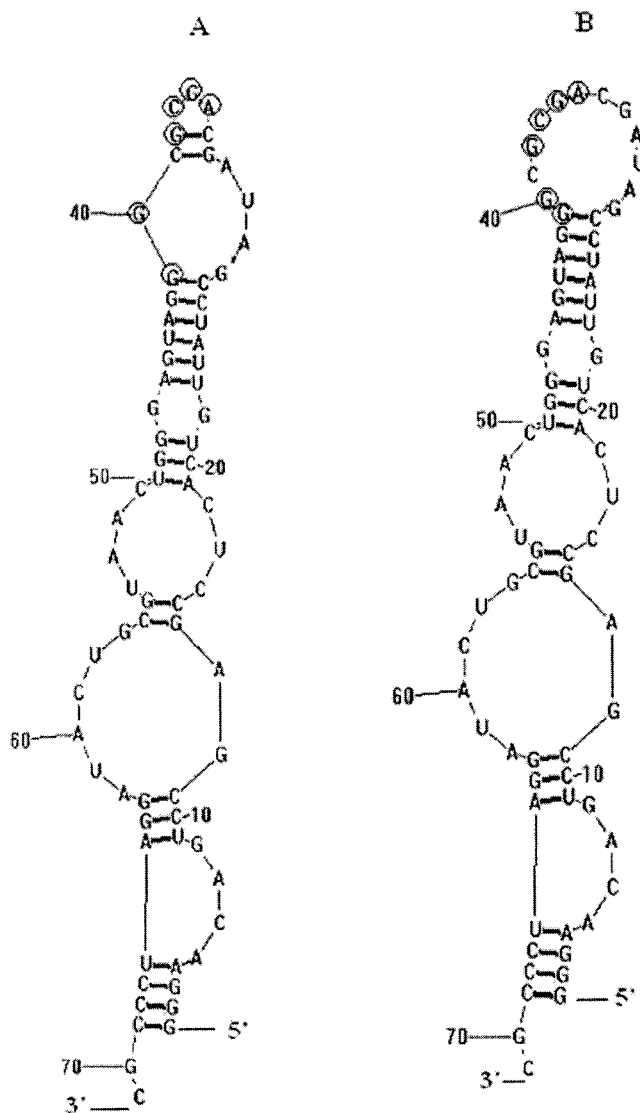


FIG. 4. Prediction of aptamer S-PS_{8,4} secondary structures. After eight rounds of SELEX selection, clone S-PS_{8,4}, which represented the most frequent RNA isolate, was selected. The secondary structures of S-PS_{8,4} (both panels A and B) were predicted by using the RNA structure program. The predicted secondary structures of S-PS_{8,4} show that the consensus AGCG-(X)-GG sequence occurs in the terminal loop of a stem-loop structure. (A) The consensus AGCG-(X)-GG sequence encircled with rings occurs in the two terminal loops of a stem-loop structure. (B) The consensus AGCG-(X)-GG sequence encircled with rings occurs in the one terminal loop of a stem-loop structure.

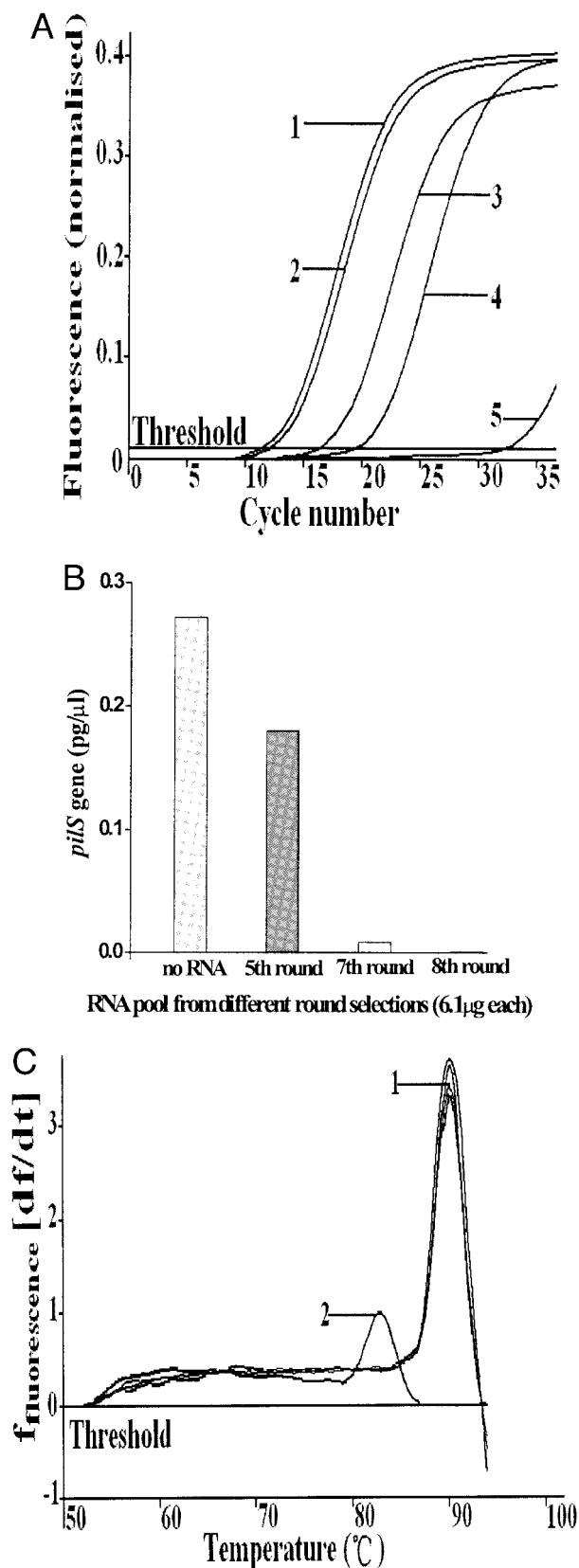


FIG. 5. Inhibition of bacterial adhesion and invasion by the RNA pools obtained at various intervals during the SELEX procedure. The assay of inhibition of bacterial adherence and invasion was performed

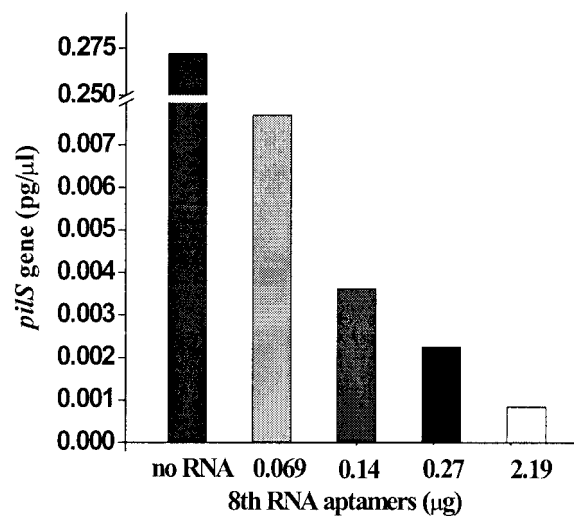


FIG. 6. Inhibition of adhesion and invasion by the eighth-round RNA pool as measured by levels of the intracellular *pilS* gene PCR product (pg/ μ l), calculated from data obtained using the real-time quantitative PCR method.

obtained and almost completely inhibited bacterial infection (Fig. 6).

Selected RNA aptamer S-PS_{8.4} significantly inhibited *S. enterica* serovar Typhi infection of human monocytes. The mixed-infection assay (Fig. 7A) and the FQ-PCR assay (Fig. 7B) showed that *S. enterica* serovar Typhi A21-6 (*pil*⁺) entered THP-1 cells to an extent greater than that of *S. enterica* serovar Typhi *pilS*::Km^r. PCR products were quantitated by measurement of the density levels of strain-specific PCR products after agarose gel electrophoresis, and the results were the same (data not shown). By the mixed-infection assay, the entry level of the *pilS* mutant was about half that of the *pil*⁺ strain. In the FQ-PCR assay, however, the entry of the *pilS* mutant was reduced to a greater extent. Thus, 19 rounds of PCR were required to achieve a fluorescence level of 0.2 for the *pil*⁺ strain but 23 rounds were needed to attain the same fluorescence level for the *pilS* mutant. The data suggest that the intracellular *pilS* DNA concentration of the *pilS* mutant was present at a level only 6 to 7% of that of the *pil*⁺ strain. There are two possibilities leading to this result. One is that the invasion of the *pil*⁺ strain induced cell ruffles neighboring the *pilS* mutant bacteria. As the type IVB pili are known to medi-

as described in Materials and Methods. (A) Inhibition of adhesion and invasion by various RNA pools as shown by real-time FQ-PCR amplification curves. The *pil*⁺ strain *S. enterica* serovar Typhi A21-6 was used at a 12:1 ratio of bacterial cells to eukaryotic cells. Curves: 1, *S. enterica* serovar Typhi A21-6 control (no RNA); 2, the fifth RNA pool; 3, the seventh RNA pool; 4, the eighth RNA pool; 5, control (THP-1 cells only). (B) Inhibition of adhesion and invasion by the various RNA pools as shown by quantitation of the cell-associated *pilS* gene PCR product (pg/ μ l), calculated with data from the real-time quantitative PCR method. (C) Melting curves of PCR products suggesting that no unspecific PCR products were formed in the PCRs. Curves: 1, real-time melting temperatures of RNA pools from the fifth, seventh, and eighth rounds of amplification; 2, melting temperature of PCR products from the control (THP-1 cells only) in the real-time FQ-PCR.

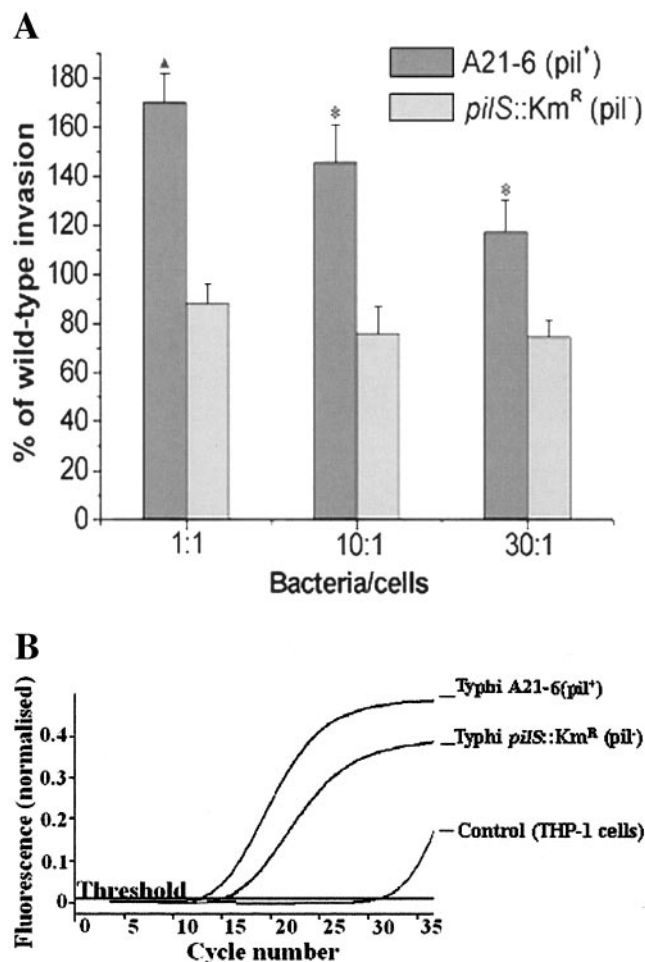


FIG. 7. Adhesion to and invasion of THP-1 cells by *S. enterica* serovar Typhi A21-6 (*pil*⁺) and *S. enterica* serovar Typhi *pilS*::*Km*^R using both mixed-infection and real-time quantitative PCR assays. (A) Adhesion to and invasion of THP-1 cells by *S. enterica* serovar Typhi A21-6 (*pil*⁺) and *S. enterica* serovar Typhi *pilS*::*Km*^R using the mixed-infection assay. A21-6 (*pil*⁺) versus *pilS*::*Km*^R (*pil*⁻) ▲, $P < 0.01$ (bacterium/cell ratio: 1:1); *, $P < 0.05$ (bacterium/cell ratios: 10:1 and 30:1). (B) Adhesion to and invasion of THP-1 cells by *S. enterica* serovar Typhi A21-6 (*pil*⁺) and *S. enterica* serovar Typhi *pilS*::*Km*^R using the real-time quantitative PCR assay. The threshold cycle (Ct) is the cycle at which there is a significant increase in fluorescence, and this value is associated with exponential growth of the PCR product during the log-linear phase. Generally, the more original the DNA template is, the fewer the threshold cycles required.

ate bacterial self-association (15), another possibility is that enmeshment of the *pilS* mutant by the accompanying *pil*⁺ strain in the mixed-infection assay yields artificially high levels of *pilS* mutant entry levels. The results of experiments comparing mixed and individual infections using a gentamicin assay also indicated that the *pilS* mutant could accompany the *pilS*⁺ strain in the mixed-infection assay (data not shown). This

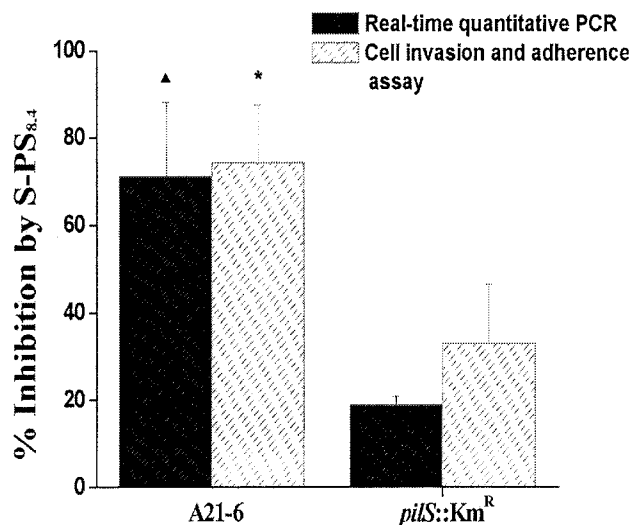


FIG. 8. S-PS_{8.4} inhibits cell invasion by *S. enterica* serovar Typhi, as shown by both real-time quantitative PCR and cell invasion and adherence assays. S-PS_{8.4} inhibited THP-1 cell invasion by *S. enterica* serovar Typhi A21-6 by 71% (real-time quantitative PCR) or 74% (cell invasion and adherence assay) and THP-1 cell invasion by the *S. enterica* serovar Typhi *pilS*::*Km*^R strain by 19% or 33%, respectively. In each assay, three experiments were carried out and each was performed in triplicate. The ratio of inhibition by S-PS_{8.4} between A21-6 (*pil*⁺) and *pilS*::*Km*^R (*pil*⁻) was statistically significant (▲, $P < 0.01$ in the real-time quantitative PCR; *, $P < 0.05$ in the cell invasion and adherence assay).

concern is absent with the FQ-PCR assay, where single bacterial strains are used.

The effects of the selected RNA aptamer S-PS_{8.4} alone on *S. enterica* serovar Typhi A21-6 and *S. enterica* serovar Typhi *pilS*::*Km*^R cell infection were determined with real-time quantitative PCR and the mixed-infection assays (Fig. 8). The results showed that 2.0 μg of RNA aptamer S-PS_{8.4} effected ca. 71% inhibition of cell invasion by *pil*⁺ *S. enterica* serovar Typhi A21-6 but only ca. 19% inhibition in the case of the *pilS*::*Km*^R mutant. These results suggested that the selected aptamer, S-PS_{8.4}, alone inhibited *S. enterica* serovar Typhi invasion of monocytes by specifically binding to type IVB pili. However, S-PS_{8.4} still showed a mild inhibitory effect on the entry of the *pilS*::*Km*^R mutant strain. It is possible that S-PS_{8.4} had a low affinity for an unknown component in the *pilS*::*Km*^R strain and therefore caused a mild reduction in cell adhesion and invasion. Results from both real-time quantitative PCR analysis and the mixed-infection assay were consistent, and we conclude that the real-time quantitative PCR analysis fairly reflects the effects of the aptamer on *S. enterica* serovar Typhi invasion.

Determination of the affinity of binding of RNA aptamers to target protein. In order to determine the binding affinities of aptamers, nitrocellulose filter-binding assays were performed. Binding reactions were carried out with a constant concentration of RNA aptamer, and the concentration of pre-PilS-GST or GST protein was titrated from 0.1 nM to 1 nM (Fig. 9). Binding curves of selected aptamers and pre-PilS showed that affinities were increased in a pre-PilS protein concentration-dependent manner and that the affinity of RNA binding to

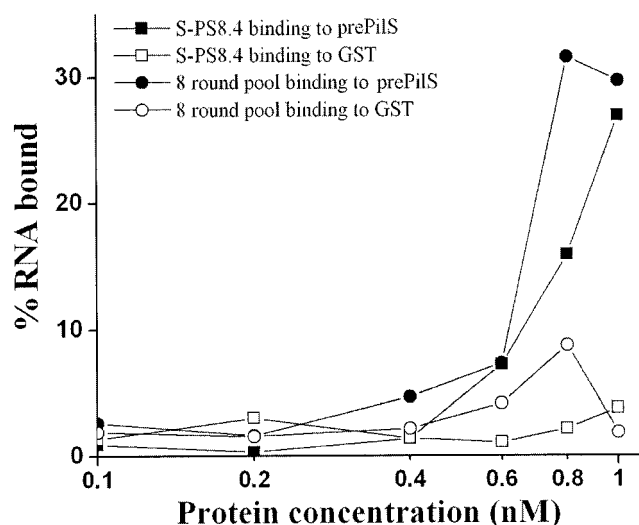


FIG. 9. Pre-PilS-binding curves of selected aptamers. Proteins were incubated with labeled RNA aptamer S-PS_{8.4} or the eighth-round labeled RNA pool. Bound aptamers were quantitated by scintillation counting. The levels of RNA bound by the pre-PilS protein were determined by subtracting the levels of RNA bound by GST protein from the levels of RNA bound by the pre-PilS-GST fusion protein. Both S-PS_{8.4} and the eighth-round pool bound strongly to pre-PilS (the K_d values were 8.56 nM and 6.08 nM, respectively) but bound weakly to GST (the K_d values were 98.13 nM and 119.28 nM, respectively).

pre-PilS was much higher than the affinity of binding to GST (Fig. 9). The K_d values of binding to pre-PilS of the eighth-round RNA aptamer pool and of clone S-PS_{8.4} alone were 6.08 nM and 8.56 nM, respectively, while the K_d values of binding to GST were 98.13 nM and 119.28 nM, respectively. Our experimental results have provided evidence of strong binding and interaction between the aptamers and PilS protein and very weak binding and interaction between the aptamers and GST protein (Fig. 9). In addition, the binding affinity of the eighth-round RNA pool was slightly higher than that of the S-PS_{8.4} aptamer alone (Fig. 9).

DISCUSSION

S. enterica serovar Typhi contains a type IVB *pil* operon that is absent in *S. enterica* serovar Typhimurium. Moreover, the type IVB pilus operon is confined to *S. enterica* serovar Typhi and a few other human-invasive strains such as *S. enterica* serovars Paratyphi C and Dublin (14, 26). The type IVB pilus-mediated events may be important in the mediation of enteric fever in humans as elements of pathogenicity required for the development of epidemics of typhoid fever.

RNA aptamers could be used as potential agents against bacterial invasion and pathogenesis. SELEX approaches have been used in the identification of RNA aptamers used to determine the toxicity of bacteria to host cells (20, 25). In addition, in vitro selection of RNA ligands that block adhesion to and invasion of monkey kidney epithelial cells by *Trypanosoma cruzi* has also been described (30) and the aptamers were successfully used to inhibit invasion by the parasite. However, selection of nucleic acids directly by affinity for bacterial proteins involved in bacterial invasion has not been reported. *S.*

enterica serovar Typhi remains epidemic in developing countries, with an estimated incidence of 33 million cases each year (27). For patients with typhoid fever, administration of an effective antibiotic is essential. However, the development of bacterial resistance to antibiotics such as chloramphenicol, ampicillin, and trimethoprim in epidemic strains is a major concern (27). RNA aptamers against type IVB pili of *S. enterica* serovar Typhi generated from SELEX selection may be further developed into new antibacterial agents with the potential to overcome resistance issues, since these RNA aptamers are different from antibiotics in terms of structure and function. In addition, these molecules may inhibit bacterial pathogenesis prior to eukaryotic cell invasion, perhaps by interfering with bacterial aggregation or directly by prohibiting bacterial entry.

Since the first description of the in vitro selection of novel ligands from combinatorial nucleic acid libraries by SELEX, much effort has been devoted to optimization of the methodology. In this study, we used Sepharose 4B instead of nitrocellulose as the selection matrix. In order to increase the specificity of selected RNA aptamers, we have utilized a combinatorial library approach by pretreating RNA aptamers with GST-Sepharose 4B before each round of SELEX selection, from the third round to the eighth and final round. Although this combinatorial library approach has been successfully used to isolate aptamers blocking ligand-receptor interactions between eukaryotic cells and viruses (2, 5, 8) or between eukaryotic cells themselves (17, 22, 29), the technique has not been applied to the selection of ligands blocking an interaction between bacterial and eukaryotic cells. Our studies indicate that the SELEX approach is a useful tool to study the roles of bacterial proteins in pathogenesis and invasion of host cells, as well as to provide insights into the mechanisms of pathogen-host cell interactions.

Our data show that the selected aptamer, S-PS_{8.4}, had the highest ratio from eight rounds of selection and a sequence that was identical to that of 9 of 14 individual clones. These findings suggested that aptamer S-PS_{8.4} should have a high affinity for the type IVB pilus protein. In the selection procedures, the RNA aptamers bound to pre-PilS competitively. Only those high-affinity aptamers had competitive advantages and survived in the final RNA pool. The aptamer with the highest frequency of occurrence in the final selected RNA pool may potentially bind to pre-PilS protein the most tightly. From our experimental results (Fig. 9), total RNA pools which contained an aptamer mixture had more binding affinity than aptamer S-PS_{8.4} alone. (The K_d values of binding to pre-PilS of the eighth-round RNA aptamer pool and of clone S-PS_{8.4} alone were 6.08 nM and 8.56 nM, respectively.) These results suggested that, except for aptamer S-PS_{8.4} alone, the other aptamers from the eighth-round selection might also have some inhibition efficiency but should have less inhibition efficiency than aptamer S-PS_{8.4}.

A consensus sequence (present eight times in the 14 clones) within individual RNA aptamers was AGCG(X)GG, and this consensus region is located in the terminal loop of the predicted S-PS_{8.4} secondary structure, suggesting that this stem-loop might be the site of binding to the target protein. Several of the other six aptamers (Table 1) without the AGCG(X)GG sequence also have stem-loop structures in predicted secondary structures; however, these stem-loops appeared to occur

only once and were not present in other aptamers and therefore these stem-loops might have a lower affinity for the pre-PilS protein. In our work, we focused on aptamer S-PS_{8,4}, which had the highest frequency of occurrence because of its high affinity for pre-PilS protein.

To identify the biological activities, and substrate affinity, of an RNA aptamer, a real-time quantitative PCR assay for quantification of bacterial adhesion and invasion was used in this work. After interaction with the RNA aptamer, a reduction of cell invasion resulted. Intracellular bacteria were quantitated by real-time PCR. Although killed extracellular bacteria may not have been completely removed, the technique is useful to measure and quantify cell invasion effects and eliminates the need for microscopic enumeration of adherent and invasive bacteria. In our studies, the results from PCR assays were consistent with those from the classical cell invasion and adherence assay. Naguleswaran et al. (16) compared the real-time quantitative PCR assay with other adhesion-invasion assays, and those results also showed the reliability of the method.

Two or three of the most efficient aptamers together to inhibit cell invasion by *S. enterica* serovar Typhi perhaps might be the perfect therapeutic agents. However, selecting several of the most efficient aptamers requires further affinity assays and biological function assays, and we have found dramatically efficient inhibition of cell invasion by *S. enterica* serovar Typhi in the high-affinity aptamer pool (the final selected RNA pool). In our study, aptamer S-PS_{8,4} was found to inhibit *Salmonella* invasion of human monocytic cells significantly.

In summary, our results suggest that aptamer S-PS_{8,4}, selected here for affinity for type IVB pili, has strong potential both as an antagonist against *S. enterica* serovar Typhi invasion of human monocytic cells and as a tool to analyze the interactions of bacterial type IVB pili with host cells and examine important aspects of bacterial pathogenesis.

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