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

Qin Pan,1 Xiao-Lian Zhang,1,2,3* Hong-Yan Wu,1 Pan-Wen He,1 Fubin Wang,1 Ming-Sheng Zhang,4 Jian-Ming Hu,2 Bing Xia,4 and Jianguo Wu3

Department of Immunology1 and Hubei Province Key Laboratory of Allergy and Immunology,2 Wuhan University School of Medicine, and Zhongnan Hospital, Wuhan University,4 Wuhan 430071, and State Key Laboratory of Virology, Wuhan University, Wuhan 430072,3 People’s Republic of China

Received 21 May 2005/Returned for modification 5 July 2005/Accepted 15 July 2005

*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 pilS 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 (pilS::Km) into human THP-1 monocytes was greater than that of a nonpiliated *S. enterica* serovar Typhi pilS::Km (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-PS8.4) as a type IVB pilus-specific ligand and further found that the selected aptamer (S-PS8.4) 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 *Kd* value was determined to be 8.56 nM for the S-PS8.4 aptamer alone. As an example of an aptamer against type IVB pili of *S. enterica* serovar Typhi, the aptamer S-PS8.4 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 pilus 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...
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 pairs can occur in ssRNA but only G-C and A-U pairs 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, monocytic/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 pli and then identified 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-PS8.4A) as a ligand of the *S. enterica* serovar Typhi type IVB pli and found it significantly inhibited the entry of the pilated strain (but not that of the nonpiliated strain) into human THP-1 cells.

**MATERIALS AND METHODS**

**Materials.** *E. coli* DH5α and BL21(DE3)plyS were used as described previously (37). *S. enterica* serovar Typhi A21-6 (pil+ / pil−) (37) was generated by inserting a tac promoter between pliM and pliN with the 575-nucleotide pliM-pliN intergenic sequence removed. Therefore, transcription of the pliN through pliV 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 the expression of the pilM-pliN operon. The nonpiliated strain *S. enterica* serovar Typhi pilS::Km (37) was generated by inserting a kanamycin resistance gene into the pilS gene so that the disruption of the pil 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 (37) for 14 to 16 h at 30°C (37). Solid medium contained 1.5% (wt/vol) agar.

**Expression and purification of pre-PilS-glutathione S-transferase (GST) fusion protein.** The PCR product of the *ps8* 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 pGEX8S, in which pilS was fused in frame with the gene for GST. pGEX8S was transformed into *E. coli* BL21(DE3)plyS. 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–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-μl reaction mixture (2 μg of 5′-μg DNA, 0.5 μM UTP, 0.5 μM GTP, 0.5 μM ATP, 0.5 μM TTP, 20 μl × 100 μl transcription buffer [Promega], 10 mM dithiothreitol [DTT], 100 μM Rnasin, 40 U T7 RNA polymerase), and the transcribed RNAs were purified using an Rnai Kit (Qiagen). To initiate in vitro selection, purified random RNAs were incubated in binding buffer (25 μM 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 1 M 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 Rnai 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-μl reaction mixture (2 μg of 5′-μg DNA, 0.5 μM UTP, 0.5 μM ATP, 0.5 μM GTP, 0.5 μM CTP, 20 μl × 100 μl 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-μl reaction volumes (1 μg DNA, 12.5 μM UTP, 0.5 μM ATP, 0.5 μM GTP, 0.5 μM CTP, 50 μl [α-32P]UTP, 4 μl 5′ transcripton 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* DH5α. 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 adhesion and invasion assays as previously described (23). THP-1 cells were grown 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 or Typhi A21-6 (pil−/ pil−) 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 μg/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 10 min and enumerated on LB agar plates and eukaryotic cells enumerated with kanamycin (both the *S. enterica* serovar Typhi A21-6 wild-type strain and the *S. enterica* serovar Typhi pilS mutant were Km). Because mixtures of the wild-type and mutant strains *S. enterica* serovar Typhi A21-6 (pil−/ km) and *S. enterica* serovar Typhi pilS::km 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 × 105 to 1 × 106/ml. *S. enterica* serovar Typhi strains (*S. enterica* serovar Typhi A21-6 and *S. enterica* serovar Typhi pilS::km) 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 μg RNA aptamer S-PS8.4A in 100 μl diethyl pyrocarbonate-treated PBS at 20 to 25°C for 15 min. The treated bacteria were added to THP-1 cells (2.5 × 105 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 × g for 10 min and washed thrice with PBS. Gentamicin (200 μg/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 no (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-PS8.4A – bacterial number with S-PS8.4A)/bacterial number with no S-PS8.4A] × 100%.
Typhi

transcription with 32P-labeled UTP, nucleoside triphosphates (GTP, CTP, UTP), and T7 RNA polymerase. Each 20-μl 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. Disociation constants were determined as previously described (10, 24). The data points of binding curves were fitted to the equation \( F = P(\frac{K_d}{K_d + I}) \) 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 pilus 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 liquid 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-PS8.4 secondary-structure prediction. After eight rounds of selection, 14 individual clones that bound to pre-PilS–GST–Sepharose 4B beads were selected and sequenced. The nucleotide sequences of the aptamers were determined by sequencing and the secondary structures of S-PS8.4 were predicted by Mfold. The results are shown in Fig. 3.
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 was concentration dependent (Fig. 6). With the addition of 6.1 $\mu$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

### TABLE 1. Aptamers selected by the SELEX procedure

<table>
<thead>
<tr>
<th>Aptamer</th>
<th>Frequency</th>
<th>RNA aptamer sequence (N 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-PS$_{8.4}$</td>
<td>5/14</td>
<td>UCAACUG UUAUCCGAUAGCAGCG G GAUGA</td>
</tr>
<tr>
<td>S-PS$_{8.3}$</td>
<td>2/14</td>
<td>A UUA CC UA G A G C G GG GAUA AAA GUA AGG G U U</td>
</tr>
<tr>
<td>S-PS$_{8.2}$</td>
<td>1/14</td>
<td>CA U CG A GG A GG C G GG UAAUUCG A U G AGU U</td>
</tr>
<tr>
<td>S-PS$_{8.5}$</td>
<td>1/14</td>
<td>A G A G U A C G A G C G GG GAUA GUAUCCG GUGGAU</td>
</tr>
<tr>
<td>S-PS$_{8.6}$</td>
<td>1/14</td>
<td>GAAGGGGU CC G GCUCC G G GG GAUGA G U UCAG GDU</td>
</tr>
<tr>
<td>S-PS$_{8.0}$</td>
<td>1/14</td>
<td>A GCUC AG CG G GGG GCG AC GG U G G U G GDU U</td>
</tr>
<tr>
<td>S-PS$_{8.7}$</td>
<td>1/14</td>
<td>UAG CG G G A G CUUGGACCUGG G G G UCGCGGC</td>
</tr>
<tr>
<td>S-PS$_{8.1}$</td>
<td>1/14</td>
<td>UUGG GAU C A GCUC GGCGC U G G AGGA G GG G C</td>
</tr>
<tr>
<td>S-PS$_{8.8}$</td>
<td>1/14</td>
<td>AAUUGG AU C A AUG GGCGCA A C AG G G A G U UAGC</td>
</tr>
</tbody>
</table>

* Consensus regions within the 14 individual aptamers are underlined.

![FIG. 4. Prediction of aptamer S-PS$_{8.4}$ secondary structures.](image-url)
obtained and almost completely inhibited bacterial infection (Fig. 6).

Selected RNA aptamer S-PS8.4 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+/H11001) entered THP-1 cells to an extent greater than that of S. enterica serovar Typhi pilS::Kmr. 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+/H11001 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+/H11001 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+/H11001 strain. There are two possibilities leading to this result. One is that the invasion of the pil+/H11001 strain induced cell ruffles neighboring the pilS mutant bacteria. As the type IVB pili are known to medi-
ate bacterial self-association (15), another possibility is that enmeshment of the pilS mutant by the accompanying pilS/H11001 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/H11001 strain in the mixed-infection assay (data not shown). This concern is absent with the FQ-PCR assay, where single bacterial strains are used.

The effects of the selected RNA aptamer S-PS₈₄ alone on S. enterica serovar Typhi A21-6 and S. enterica serovar Typhi pilS::Kmr 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-PS₈₄ effected ca. 71% inhibition of cell invasion by pilS/H11001 S. enterica serovar Typhi A21-6 but only ca. 19% inhibition in the case of the pilS::Kmr mutant. These results suggested that the selected aptamer, S-PS₈₄, alone inhibited S. enterica serovar Typhi invasion of monocytes by specifically binding to type IVB pili. However, S-PS₈₄ still showed a mild inhibitory effect on the entry of the pilS::Kmr mutant strain. It is possible that S-PS₈₄ had a low affinity for an unknown component in the pilS::Kmr 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
The type IVB pilus operon is confined to 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 combinational 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-PiIS 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-PiIS 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

**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 pil 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.

![Graph showing Pre-PiIS-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-PiIS protein were determined by subtracting the levels of RNA bound by GST protein from the levels of RNA bound by the pre-PiIS–GST fusion protein. Both S-PS$_{8.4}$ and the eighth-round pool bound strongly to pre-PiIS (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).](image-url)
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$_{S_{4,4}}$ 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 adhesion 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 	extit{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 	extit{S. enterica} serovar Typhi in the high-affinity aptamer pool (the final selected RNA pool). In our study, aptamer S-PS$_{S_{4,4}}$ was found to inhibit 	extit{Salmonella} invasion of human monocytic cells significantly.

In summary, our results suggest that aptamer S-PS$_{S_{4,4}}$, selected here for affinity for type IVB pili, has strong potential both as an antagonist against 	extit{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.

ACKNOWLEDGMENTS

This work was supported by grants from the National Science Foundation of China (30270076) and the Ministry of Education Scientific Research Foundation for Returned Overseas Chinese Scholars (30115303), Outstanding Young Scholars grants from Hubei Province (301153033), Outstanding Youth Scholars grants from Hubei Province (30115303), and grants from Wuhan University (301270055 and 301276010).

REFERENCES


15. White, R. R., S. Shan, C. P. Rascon, G. Shetty, M. W. Dewhurst, C. D.


