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 pililiated S. enterica serovar Typhi A21-6 (pil+ Kmr) into human THP-1 monocytic cells was greater than that of a nonpiliated S. enterica serovar Typhi pilS::Kmr (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 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
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 PiS protein.

During natural *Salmonella* bacterial infections, monocyt/macrophages serve as key effector cells of the immune response. In order to further investigate the pathogenic roles of the PiS protein of the type IVB pili and then investigated the effects of the aptamers on *S. enterica* serovar Typhi adhesion to and invasion of the human monocytic leukemic cell line THP-1. We have identified a single ssRNA aptamer (S-PS3a) as a ligand of the *S. enterica* serovar Typhi type IVB pili 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) 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 5′-nucleotide plm-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 the lac promoter. 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-Phosphate-4B. 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 to 5 μg DNA, 0.5 μM UTP, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 2 μl 5× transcription buffer [Promega], 10 mM dithiothreitol [DTT], 100 μM RNasin, 40 U T7 RNA polymerase), and the transcribed 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-PiS 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-Phosphate-4B. 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 to 5 μg DNA, 0.5 μM UTP, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 2 μl 5× 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-PiS–GST–Sepharose 4B material.

**In vitro transcription.** The RNA products of the test application and of a control in which the T7 DNA 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 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 50 μC [32P]UTP, 4 μl 5× 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* 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 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 (Gibco) 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-PiS–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. 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. 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.
Typhi/H11032) and PR, respectively. Forward primer P F2, for amplification of C3 mutant DNA, lies in the Kmr 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 (10, 24). The data points of binding curves were fitted to the equation (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 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 (>-1012 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).

**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 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 determine the effects of aptamer S-PS8.4 on 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 (>-1012 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 the pre-PilS–GST–Sepharose 4B beads were selected and sequenced.
sequenced. 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) 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-PS8.4 appeared five times in the RNA aptamer pool. The secondary structure of the S-PS8.4 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-PS8.4 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-PS8.4 RNA aptamer. These two predicted secondary structures are equally likely from the sequence analysis of aptamer S-PS8.4, 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 μg 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>
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<th>Aptamer</th>
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<th>RNA aptamer sequence (N 30)</th>
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* Consensus regions within the 14 individual aptamers are underlined.
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+ strain) 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+ 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-
ate bacterial self-association (15), another possibility is that enmeshment of the pilS mutant by the accompanying pilSH11001 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 pilSH11001 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-PS8.4 alone on S. enterica serovar Typhi A21-6 and S. enterica serovar Typhi pilS::Km cell invasion 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-PS8.4 effected ca. 71% inhibition of cell invasion by pilSH11001 S. enterica serovar Typhi A21-6 but only ca. 19% inhibition in the case of the pilS::Km mutant. These results suggested that the selected aptamer, S-PS8.4, alone inhibited S. enterica serovar Typhi invasion of monocytes by specifically binding to type IVB pili. However, S-PS8.4 still showed a mild inhibitory effect on the entry of the pilS::Km mutant strain. It is possible that S-PS8.4 had a low affinity for an unknown component in the pilS::Km 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. Typhi and a few other human-invasive strains such as 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 bacterial proteins in bacterial invasion has not been reported. S. enterica serovar Typhi contains a type IVB pil operon that is absent in S. enterica serovar Typhimurium. However, 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).

FIG. 9. Pre-PilS-binding curves of selected aptamers. Proteins were incubated with labeled RNA aptamer S-PSPS8.4 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-PS8.4 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 and interaction between the aptamers and PilS protein and very weak binding and interaction between the aptamers and GST were 98.13 nM and 119.28 nM, respectively.

Our data show that the selected aptamer, S-PS8.4, 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-PS8.4 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-PS8.4 alone. (The $K_d$ values of binding to pre-PilS of the eighth-round RNA aptamer pool and of clone S-PS8.4 alone were 6.08 nM and 8.56 nM, respectively.) These results suggested that, except for aptamer S-PS8.4 alone, the other aptamers from the eighth-round selection might also have some inhibition efficiency but should have less inhibition efficiency than aptamer S-PS8.4.

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-PS8.4 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-PiS protein. In our work, we focused on aptamer S-PS₄,₄ which had the highest frequency of occurrence because of its high affinity for pre-PiS 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-PS₄,₄ was found to inhibit Salmonella invasion of human monocytic cells significantly.

In summary, our results suggest that aptamer S-PS₄,₄, selected here for affinity for type IVB pili, has strong potential which had the highest frequency of occurrence because of its high affinity for pre-PiS protein.

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