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Targeting Essential Genes in *Salmonella enterica* Serovar Typhimurium with Antisense Peptide Nucleic Acid

Muhammad A. Soofi and Mohamed N. Seleem

Department of Comparative Pathobiology, College of Veterinary Medicine, Purdue University, West Lafayette, Indiana, USA

We investigated the capability of antisense peptide nucleic acids (PNAs) conjugated to the $(KFF)_3K$ cell-penetrating peptide to target possible essential genes (*ligA*, *rpoA*, *rpoD*, *engA*, *tsf*, and *kdtA*) in *Salmonella enterica* serovar Typhimurium and inhibit bacterial growth *in vitro* and in cell culture. All targeted PNA-based gene inhibition has shown great potency in gene expression inhibition in a sequence-specific and dose-dependent manner at micromolar concentrations. Among tested PNAs, the anti-*rpoA* and -*rpoD* PNAs showed the greatest potency.

Salmonella enterica serovar Typhimurium remains the leading cause of extraintestinal focal infection in both developing and developed countries, and it places an exceptional burden on health care institutions worldwide (4, 16). The rising incidence of multidrug-resistant (MDR) strains provides a strong impetus to develop novel methods to combat salmonellosis. Antisense oligonucleotides (ODN) have successfully been employed in down-regulating specific target genes, and they represent an innovative advancement in gene-silencing technology (5, 6). Peptide nucleic acid (PNA) is an ODN analog built off a pseudopeptide backbone (19, 20). PNA molecules demonstrate high levels of specific hybridization to complementary DNA and, after annealing, can cause steric hindrance to ribosomes and other cellular enzymes. These favorable properties besides the low toxicity have been harnessed to silence genes critical for bacterial viability, thereby inhibiting bacterial growth (1, 2, 11–14, 18, 23). Here we designed six PNA oligomers targeting six proposed essential genes in *Salmonella* (Table 1): a DNA ligase gene (*ligA*), an RNA polymerase α subunit gene (*rpoA*), an RNA polymerase sigma 70 (sigma D) factor gene (*rpoD*), a gene essential for normal ribosome maturation and cell viability (*engA*) (15), a protein chain elongation factor gene (*tsf*), and a gene responsible for fatty acid synthesis (*kdtA*) (3).

PNA-peptide conjugates were purchased from Panagene (Daejeon, South Korea). The cell-penetrating peptide (CPP) $(KFF)_3K$ (8, 10) was covalently attached to the PNA construct. The specific 11-nucleotide sequence of the PNA construct (Table 1) was chosen to be complementary to a specific region of the critical gene's mRNA, including the translation start codon and the 5′-terminal region, since this region is accessible for ribosome assembly and consistent success has been experienced targeting this region (7, 21). Antisense PNA was added to 2.7×10^4 CFU/ml *Salmonella enterica* serovar Typhimurium LT2 (ATCC 700720) culture in siliconized microcentrifuge tubes. The cultures were incubated at 37°C with shaking for 8 h. The numbers of viable bacteria were enumerated by serial dilution and counting on tryptic soy agar (TSA) plates. In at least two independent trials performed in triplicate, all antisense PNAs induced significant reduction in the growth rate of *Salmonella* (Table 2). The antisense inhibition effects observed were concentration dependent. Although significant reduction of growth rate was observed with all designed PNAs, clearance was observed only with *rpoA* and *rpoD* antisense PNAs. The lower potency in growth inhibition seen with some essential genes can be explained by partial mRNA inhibition and a requirement for greatly reduced gene expression to have an effect on growth (stringency requirement) (9). Although this inhibition

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Address correspondence to Mohamed N. Seleem, mseleem@purdue.edu.

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TABLE 1 *Salmonella* targeted genes and PNA-specific sequence

Gene	Accession no.	Sequence[a]	Proposed function
ligA	2539001	H-KFFKFFKFFK-o-catatcacacc-NH_2	Catalyzes formation of phosphodiester linkages between 5′-phosphoryl and 3′-hydroxyl groups in double-stranded DNA
rpoA	3583301	H-KFFKFFKFFK-o-cattgtgtcct-NH_2	Encodes α-subunit of RNA polymerase, which is involved in transcription of DNA into RNA
rpoD	3376381	H-KFFKFFKFFK-o-cataagacggt-NH_2	Primary sigma factors are initiation factors that promote attachment of RNA polymerase to specific initiation sites
engA	2651457	H-KFFKFFKFFK-o-catgtttaaag-NH_2	Essential GTPase; synchronizes cellular events by interacting with multiple targets with tandem G-domains
tsf	255260	H-KFFKFFKFFK-o-cattctaaaat-NH_2	Elongation factor functions during elongation stage of protein translation, forms a dimer, associates with EF-Tu-GDP complex
kdtA	3919318	H-KFFKFFKFFK-o-catagtaaata-NH_2	3-Deoxy-D-manno-octulosonic-acid transferase; catalyzes transfer of 2-keto-3-deoxy-D-manno-octulosonic acid to lipid A
Control PNA gene		H-KFFKFFKFFK-o-cataatgtccg-NH_2	

[a] Lowercase letters represent the specific 11-nucleotide sequence of the PNA construct; -o- represents linker.

TABLE 2 Susceptibility of *S. enterica* serovar Typhimurium to 20 μM PNAs after 8 h of incubation in TSB culture[a]

Antisense PNA or water	Susceptibility to 20 μM PNA	
	Log CFU	Log CFU reduction
Control	8.53 ± 0.11	0.26
Anti-*ligA*	8.11 ± 0.45	0.68*
Anti-*rpoA*	0	8.79*
Anti-*rpoD*	0	8.79*
Anti-*engA*	7.2 ± 0.10	1.59*
Anti-*tsf*	6.57 ± 0.36	2.22*
Anti-*kdtA*	5.66 ± 0.23	3.13*
Water	8.79 ± 0.04	0

[a] PNAs were added to 2.7×10^4 CFU/ml *S. enterica* serovar Typhimurium in TSB and incubated for 8 h as described in the text. Samples were serially diluted, and the numbers of CFU/ml were estimated by plating onto TSA. The CFU shown represent the means ± standard errors from at least 2 independent experiments. Statistical analysis was performed for comparisons between groups with Student's *t* tests. The statistical significance level for the experiments was defined as $P < 0.05$ (asterisks).

was not observed in bacterial cultures treated with antisense mismatched PNA (Table 3), interactions with other targets with mismatches cannot be excluded, especially at the PNA concentrations used, which also could explain the lower potency in growth inhibition seen with some essential genes. The efficacy of the PNA conjugates was also ascertained in cell culture. J774A.1 cells were infected with *S. enterica* serovar Typhimurium following the procedures described before (22). The infected cells were treated with antisense PNAs at concentrations of 5 μM and 30 μM for 4 h (Table 4). The number of viable bacterial cells was determined by lysing the J774A.1 cells and counting the number of subsequent colonies on TSA plates. The numbers of viable bacterial cells in the culture were significantly reduced in the presence of the antisense peptide-PNA conjugates compared to the numbers in cell cultures treated with the PNA control only in 30 μM concentrations.

One of the central factors limiting PNA antibacterial activity is bioavailability of the PNA inside the target bacterium, and the major obstacle to bacterial uptake of PNA is the double membrane of Gram-negative bacteria. The ability of the PNAs to cross the cytoplasmic membrane was facilitated by the CPP via the linker. However, the relatively high molecular weights and possibly the polarity account for the decreased antibacterial activity that is observed when comparing activities *in vitro* and in cell culture (17, 21). Conjugates needed to traverse two phospholipid membranes in addition to the cell membrane of the bacteria to reach the target mRNA and the introduction of these additional barriers constrained bioaccumulation of the PNA-peptide conjugates inside

TABLE 3 Concentration-dependent bactericidal antisense effects of anti-*rpoA* and anti-*rpoD*[a]

Antisense PNA or water	Effect at PNA concn of:					
	5 μM		10 μM		15 μM	
	Log CFU	Log CFU reduction	Log CFU	Log CFU reduction	Log CFU	Log CFU reduction
Anti-*rpoA*	5.74 ± 0.68	3.16*	5.24 ± 0.20	3.63*	0	8.92*
Anti-*rpoD*	6.74 ± 0.33	2.16*	4.21 ± 0.03	4.66*	0	8.92*
Control	8.90 ± 0.03	0	8.53 ± 0.11	0.34	9.01 ± 0.05	−0.09
Water	8.90 ± 0.01	0	8.87 ± 0.05	0	8.92 ± 0.05	0

[a] PNAs were incubated with $\sim 2 \times 10^4$ CFU/ml *S. enterica* serovar Typhimurium in TSB for 8 h. Samples were serially diluted, and CFU/ml were estimated by plating onto TSA. The CFU represent the means ± standard errors from at least 2 independent experiments. Statistical analysis was performed for comparisons between groups with Student's *t* tests. The statistical significance level for the experiments was defined as $P < 0.05$ (asterisks).

TABLE 4 Concentration-dependent bactericidal antisense effects of PNAs in cell culture[a]

Antisense PNA	Effect at PNA concn of:			
	5 μM		30 μM	
	Log CFU	Log CFU reduction	Log CFU	Log CFU reduction
Anti-*ligA*	3.53 ± 0.05	−0.56	3.9 ± 0.04	0.51*
Anti-*rpoA*	3.51 ± 0.14	−0.54	3.89 ± 0.09	0.52*
Anti-*rpoD*	3.02 ± 0.20	−0.05	4.00 ± 0.02	0.41*
Anti-*engA*	2.85 ± 0.09	0.12	3.84 ± 0.06	0.57*
Anti-*tsf*	2.76 ± 0.11	−0.21	3.5 ± 0.02	0.91*
Anti-*kdtA*	2.80 ± 0.19	−0.17	3.68 ± 0.03	0.73*
Control	2.97 ± 0.22	00	4.41 ± 0.03	0

[a] PNAs were incubated with *S. enterica* serovar Typhimurium-infected J774 cells for 4 h. The number of viable bacterial cells was determined by lysing the J774A.1 cells and counting the number of subsequent colonies on TSA plates. Values represent the means ± standard errors of CFU from at least 2 independent experiments. Statistical analysis was performed for comparisons between groups with Student's *t* tests. The statistical significance level for the experiments was defined as $P < 0.05$ (asterisks).

the bacterium, thereby reducing antibacterial activity compared to that in *in vitro* models. It is possible that PNA-peptide conjugates become sequestered in an endosomal pathway in the host cell and the incubation time may not be long enough for conjugates to escape this pathway and enter the bacterium. In this study, we investigated the possibility of using PNA-peptide conjugates as novel antimicrobial agents capable of challenging *S. enterica* serovar Typhimurium. By exploring the efficacy of specifically engineered conjugates both *in vitro* and in cell culture, we were able to establish that PNA-peptide conjugates represent a viable approach toward producing innovative antibacterial agents. The lower inhibition rate of growth obtained by some targets does not necessarily eliminate the putative targets that were selected. These conjugates have not been optimized, and further modifications may allow for greater antibacterial activity or lower MIC values.

PNAs targeted against essential bacterial genes enable one to find out which genes are susceptible targets for more conventional antimicrobial development. Previous studies have suggested the susceptibility of the *rpoD* gene and its products toward antimicrobial compounds and have proposed that this gene be considered a candidate for drug discovery (1, 2). Our study showed that the *rpoA* gene and its product, the α-subunit of the RNA polymerase, are also another potential targets; both are unique among bacteria and are different from the eukaryotic homolog.

This study also demonstrated the sequence-specific binding capabilities of peptide nucleic acids. The control PNA lacked sequence homology with any of the genes delineated in Table 1, and it was reported to have no significant antibacterial activity. In contrast, the PNA constructs that were directly complementary to their target genes had significant levels of bacterial inhibition, suggesting that sequence homology was necessary for PNA binding and that PNA constructs could selectively hybridize to complementary nucleic acids.

Although this work demonstrates the potential of developing PNA-peptide conjugates into a new suite of antimicrobial compounds, there are still certain advances that would allow for the optimization of this approach. In addition, further research on the safety, toxicity, and pharmacokinetic aspects would be valuable in elucidating the different characteristics of PNA-peptide treatment.

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