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A Novel Peptide Nucleic Acid against the Cytidine Monophosphate Kinase of *S. aureus* Inhibits Staphylococcal Infection *In Vivo*

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Here, we report a novel bactericidal peptide nucleic acid (PNA) that can induce the antisense effect on the cytidine monophosphate kinase (Cmk) of *Staphylococcus aureus*, a putative essential component for bacterial species. Based on the genome sequence of *S. aureus* N315, a set of PNA conjugates with a bacterial penetration peptide, (KFF)$_3$K, were synthesized to target the seven potentially essential genes (*cmk*, *deoD*, *ligA*, *smpB*, *glmU*, *pyrH*, and *ftsA*) and further evaluated for their antibacterial properties *in vitro* as well as *in vivo*. The results demonstrated that two peptide-conjugated PNAs (P-PNAs), antisense P-PNA (ASP)-cmk1 and ASP-deoD1, targeting either the *cmk* or the *deoD* genes, had the strongest inhibitory effects on the growth of *S. aureus* ATCC 29740 (a bovine mastitic milk isolate) in a dose-dependent manner. *In vivo* application of ASP-cmk1 resulted in a significant reduction of bacterial loads in mice intraperitoneally infected with a sublethal dose of *S. aureus*. Moreover, ASP-cmk1 significantly increased the survival rate of the breast-fed infant mice after intramammary infection of the lactating CD-1 mice. Taken together, our characterization of ASP-cmk1 demonstrated its bactericidal activity against *S. aureus* as well as its effectiveness *in vivo*.

INTRODUCTION

Peptide nucleic acid (PNA) is an artificially synthesized DNA mimic that forms Watson-Crick base pairs with both DNA and RNA molecules in a sequence-dependent manner.[1] Unlike naive DNA or RNA molecules, PNA has a neutral and flexible, noncyclic peptide-like backbone.[1] This unique feature provides PNA with (1) increased hybridization affinity and specificity in the presence of various ion concentrations, (2) high chemical stability at various temperature and pH, and (3) enzymatic resistance to serum proteases and nucleases. Moreover, it is known to be rarely toxic *in vivo*.[2] Therefore, PNAs complementary to certain target genes are advantageous for inducing target-specific gene silencing, especially *in vivo*.[1,3] For example, it has been reported that a 16-mer PNA can suppress cancer cells by blocking transcription of the MYCN oncogene, and the resultant DNA::PNA duplex is exceptionally stable.[3] The PNA-mediated mRNA splicing correction was also demonstrated in a mouse model of Duchenne muscular dystrophy without any *in vivo* toxic effects.[4]

In cells, PNA-induced gene silencing is achieved by targeting either DNAs (referred to as an antigene approach) or mRNAs (an antisense approach).[5] The PNAs with an antigene effect break up the DNA duplex to form a PNA:DNA triplex or double duplexes without denaturing the intact DNA duplex and interfere with replication or transcription of the target genes.[5] In contrast, the PNAs with an antisense effect can hybridize to the complementary sequences of the target mRNAs (i.e., ribosome-binding consensus mRNA sequences) and thereby inhibit translation.[5] To maximize those antigene or antisense effects of PNAs *in vivo*, however, PNAs must be delivered into cells or subcellular organelles across biological membranes, such as membrane lipid bilayers, lipopolysaccharides, and peptidoglycans.[1,2,5] Among such delivery systems that enhance cellular uptake of PNAs across biological membranes, the peptide-mediated delivery system with a synthetic, small cationic, bacterial penetration peptide motif, (KFF)$_3$K, has been well established.[6] Indeed, previous *in vitro* and *in vivo* studies demonstrated more effective and safer uptake of (KFF)$_3$K peptide-conjugated PNAs (P-PNAs) into both eukaryotic and prokaryotic cells than that of unconjugated PNAs.[6,7] Although the mechanisms behind the cellular uptake of P-PNAs remain unclear, a recent study demonstrated that SbmA, an inner membrane peptide transporter, is involved in the cellular uptake of (KFF)$_3$K P-PNA conjugates.[8]

The emergence and rapid spread of multidrug-resistant bacteria as well as the slow progress in discovering new classes of antimicrobials pose threats to public health worldwide. Recently, antisense P-PNAs targeting essential genes have been described as alternative antibacterial agents against certain bacterial pathogens, including *Escherichia coli*,[9,10] *Staphylococcus aureus*,[11–15] *Klebsiella pneumoniae*,[16] and *Pseudomonas aeruginosa*.[17] Studies have demonstrated that the antisense P-PNAs can block the expression of the essential target genes (i.e., *gyrA* for DNA replication, *ftsZ* for cell division, *acpP* for fatty

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Table 1. Antisense P-PNA Oligomers in This Study

Name	Target Gene	Protein	Function	5′ to 3′ Sequences (Location)
ASP-cmk1	*cmk*	cystidine monophosphate kinase	ATP + dCMP/dUMP → ADP + dCDT/dUDP	$(KFF)_3$K-L-tcatgcgtca (−6 to +4)
ASP-deoD1	*deoD*	purine nucleoside phosporylase	nucleotide synthesis (inosine)	$(KFF)_3$K-L-tcatttgtta (−6 to +4)
ASP-ftsA2	*ftsA*	cell division protein FtsA	early component of the Z-ring required for cell division	$(KFF)_3$K-L-catagataggcag (−10 to +2)
ASP-glmU1	*glmU*	UDP-N-acetyl glucosamine pyro-phosphorylase	acetyl transferase or uridyl transferase essential for bacterial cell wall	$(KFF)_3$K-L-gcatgaacat (−6 to +3)
ASP-ligA1	*ligA*	DNA ligase	ligation	$(KFF)_3$K-L-ccatccctta (−6 to +4)
ASP-pryH1	*pryH*	uridylate kinase	UMP kinase catalyzing UMP phosphorylation	$(KFF)_3$K-L-ccattttctt (−6 to +4)
ASP-smpB1	*smpB*	SsrA (small stable RNA A)-binding protein	required for recycling ribosomes	$(KFF)_3$K-L-tcatactcac (−10 to −1)

acid synthesis) and inhibit bacterial growth in both sequence- and dose-dependent manners.[13,17] In this study, PNAs conjugated with a bacterial penetration peptide, $(KFF)_3K$, were designed to target genes potentially essential for bacterial survival and further evaluated for their antibacterial properties against *S. aureus* strains, including *S. aureus* ATCC 29740[18] and the methicillin-resistant *S. aureus* (MRSA) N315.[19] *S. aureus* ATCC 29740 was chosen in this study because it was originally isolated from a cow with a clinical case of mastitis,[18] and its murine infection model systems were well established, although it was not multidrug resistant.

RESULTS

Identification of Two P-PNAs, Antisense P-PNA (ASP)-cmk1 and ASP-deoD1, with Strong Antibacterial Activity against *S. aureus* ATCC 29740

The antibacterial activities of the synthesized P-PNAs, targeting the seven potentially essential genes (Table 1), were examined against *S. aureus* ATCC 29740 by a standard spot assay (see Materials and Methods). Among the seven P-PNAs that are fully complementary to the target sequences of individual genes (Table 1), two novel P-PNAs, ASP-cmk1 and ASP-deoD1, exhibited the strongest growth inhibitory effect at a concentration of 40 μM (Figure 1). Indeed, no viable colonies of *S. aureus* were recovered at 8 h after treatment with either ASP-cmk1 or ASP-deoD1 (Figure 1). Although the magnitude of bactericidal activity differed from those of ASP-cmk1 and ASP-deoD1, some P-PNAs (designated as ASP-ligA1, ASP-pryH1, and ASP-smpB1) displayed intermediate growth inhibitory effects (Figure 1). No obvious growth inhibition was observed in

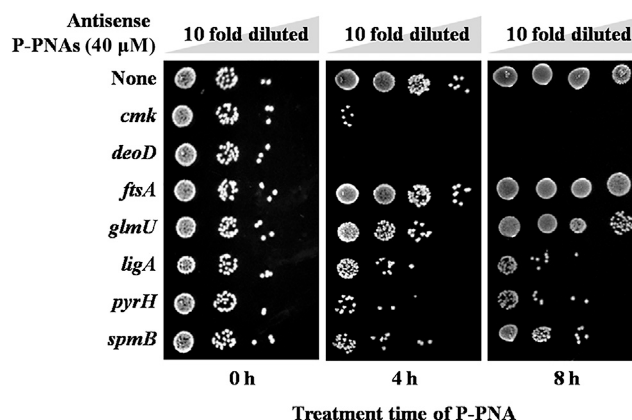

Figure 1. Antibacterial Activity of P-PNAs against *S. aureus* ATCC 29740
S. aureus ATCC 29740 of 6.0 ± 1.6 × 10^4 CFU mL^{-1} was cultured with 40 μM of each P-PNA. After 4 or 8 h of incubation, the bacterial culture was serially diluted, and 5 μL of each diluent was dropped on MH agar plates. The plates were further incubated at 37°C for 24 h. Experiments were performed in triplicate, and a representative plate is shown here.

S. aureus treated with either ASP-ftsA2 or ASP-glmU1 (Figure 1). As shown in Figure S1, we further tested the antibacterial activities of P-PNAs with additional *S. aureus* strains, including MRSA N315, three Korean bovine mastitis isolates of *S. aureus*, and other bacterial species. Consistently, both ASP-cmk1 and ASP-deoD1 showed the strong antibacterial activity (Figure S1). These results imply that both ASP-cmk1 and ASP-deoD1 are most effective for blocking their target genes (*cmk* and *deoD*, respectively) in *S. aureus* and thereby exert bactericidal activities.

Determination of the Minimal Effective Concentrations (MECs) of Both ASP-cmk1 and ASP-deoD1

Since both ASP-cmk1 and ASP-deoD1 were effective for killing *S. aureus* ATCC 29740 as early as 4 h after treatment (Figure 1), we measured their MECs by direct colony counting of bacterial cultures at 4 h after treatment with various concentrations of each P-PNA up to 40 μM (Figure 2). As expected, both ASP-cmk1 and ASP-deoD1 inhibited bacterial growth in a dose-dependent manner, which began to be effective at a concentration of 10 μM (Figure 2). Consistent with the results from the preliminary spot assay (Figure 1), the greatest inhibitory effects were observed at a concentration of 40 μM for each P-PNA (Figure 2). In this study, however, 20 μM ASP-cmk1 or ASP-deoD1 were chosen for further analyses because we observed no significant differences in their biological activities at the concentrations of 20 and 40 μM.

Antisense Effects of ASP-cmk1 and ASP-deoD1 on the *cmk* and *deoD* Genes

To investigate the mechanisms behind the growth inhibitory effects of the P-PNAs in *S. aureus* ATCC 29740, we checked the mRNA expression levels of *cmk*, *deoD*, and *gyrB* (as a control). The mRNA expression levels of both *cmk* and *deoD* were not significantly changed after treatment with each P-PNA, suggesting that both ASP-cmk1 and

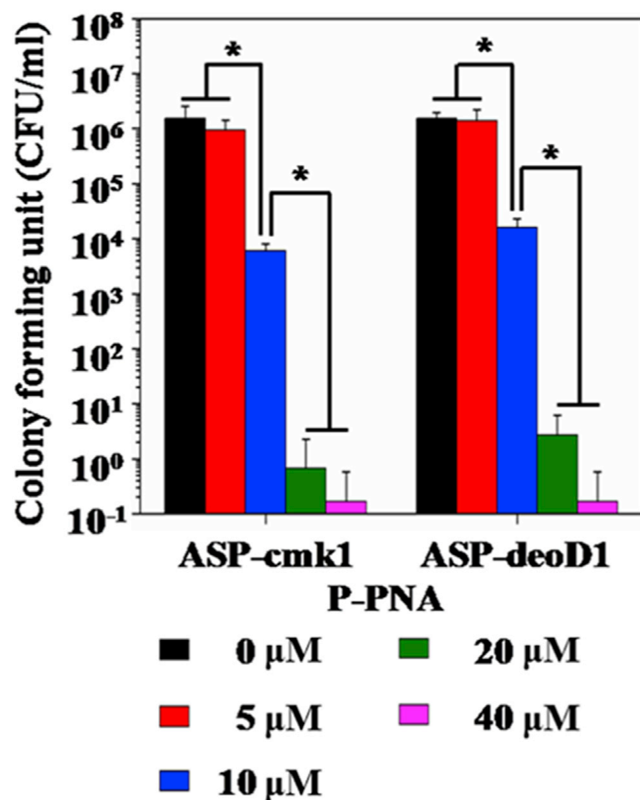

Figure 2. Determination of the Minimal Effective Concentrations of Novel P-PNAs, ASP-cmk1, and ASP-deoD1

S. aureus ATCC 29740 at approximately 5.0×10^4 CFU mL^{-1} was cultured for 4 h with the indicated concentrations of each P-PNA, and the growth inhibitory effects were monitored quantitatively by direct plating (see Materials and Methods). The values are presented as mean ± SD (n = 3). The asterisk indicates that the values are significantly different with a p value of < 0.05.

ASP-deoD1 had no antigene effects in *S. aureus* ATCC 29740 (Figure 3A). To analyze possible changes in the protein levels, western blot analyses were initially performed using a Cmk-specific polyclonal antibody that had been raised in rabbits with the putative immunodominant epitope. Unfortunately, we failed to do so due to the low specificities of the polyclonal antibody. Alternatively, we constructed the *cmk* or *deoD* promoter and *lacZ* translational fusion systems in *E. coli* DH5α to analyze their translation efficacy using a β-galactosidase assay (see Materials and Methods). The results demonstrated that both ASP-cmk1 and ASP-deoD1 inhibit the translation of a downstream gene, *lacZ*, in a dose-dependent manner, even though the bacterial count did not differ significantly (Figures 3B and 3C). In contrast, ASP-deoD1 had no effect on the expression of the *cmk* promoter-*lacZ* translational fusion construct, which was also true in the opposite case (Figure 3B). Likewise, *S. aureus* RN4220 harboring pMKcmk or pMKdeoD showed reduced β-galactosidase activity (approximately 40%) when treated with P-PNAs (Figure 3C). Although our *lacZ* translation fusion systems in both *E. coli* and *S. aureus* RN4220 reflect the transcription expression of both *cmk* and *deoD* genes as well as their translation expression levels (Figures 3B and 3C), the previous quantitative real-time PCR results revealed that the transcription of both genes were not changed by ASP-cmk1 or ASP-deoD1 (Figure 3A). Therefore, these results imply that P-PNAs in this study function through a sequence-specific antisense mechanism rather than an antigene mechanism.

In Vivo Protection by ASP-cmk1 against Staphylococcal Infections in the Murine Intraperitoneal and Intramammary Infection Models

To investigate the potential *in vivo* effects of ASP-cmk1, mice were intraperitoneally infected with *S. aureus* ATCC 29740 with or without ASP-cmk1. The results showed that the treatment with ASP-cmk1 significantly reduced the number of bacteria in both peritoneal fluid and kidneys of the infected mice ($p < 0.05$; Figure 4). Indeed, three to four magnitudes of bacterial reduction on the logarithmic scale were observed in the ASP-cmk1-treated group, compared to the control group (Figure 4). Consistently, our results demonstrated that the treatments of ASP-cmk1 dramatically increased the survival rate of the infant mice when a murine intramammary infection model was applied (see Materials and Methods). As shown in Table 2, saline-treated suckling mice died within 24 h after breast feeding from the lactating CD-1 mice intramammarily infected with *S. aureus* ATCC 29740. On the other hand, surprisingly, ASP-cmk1 treatment was able to protect all infant mice from staphylococcal infection by suckling (Table 2). Although we did not measure the tissue distribution of ASP-cmk1 after its intramammary injection, these results suggest that ASP-cmk1 is effective *in vivo* and thereby ameliorates bacterial infectivity, at least in mice infected with *S. aureus* ATCC 29740.

Interestingly, the intravenous injection of ASP-cmk1 (1,000 nmol) into the CD-1 mice did not induce any behavioral changes nor tissue damages in various organs, implying no toxicity of ASP-cmk1 in mice at least under the conditions evaluated in this study (Figure S2).

DISCUSSION

In this study, we discovered two antibacterial P-PNAs, ASP-cmk1 and ASP-deoD1, which have antisense effects on the previously defined and potentially essential genes among bacterial species, *cmk* and *deoD*, in a sequence-dependent manner.[20] Further characterization of ASP-cmk1 demonstrated its effectiveness *in vitro* and *in vivo*. This is the first demonstration of a *cmk*-targeting antisense P-PNA with antibacterial activity as well as its effectiveness *in vivo* against *S. aureus*.

Among the seven P-PNAs evaluated in this study, two (ASP-cmk1 and ASP-deoD1) showed strong antibacterial activity against *S. aureus*. Because all of the targeted genes are potentially essential for survival and conserved across diverse bacterial species,[20] the reason(s) why some differences in antibacterial activity were observed among our designed P-PNAs is unclear. However, a recent study demonstrated that the inhibition of bacterial growth by RNA silencing of some essential genes depends on the growth requirement

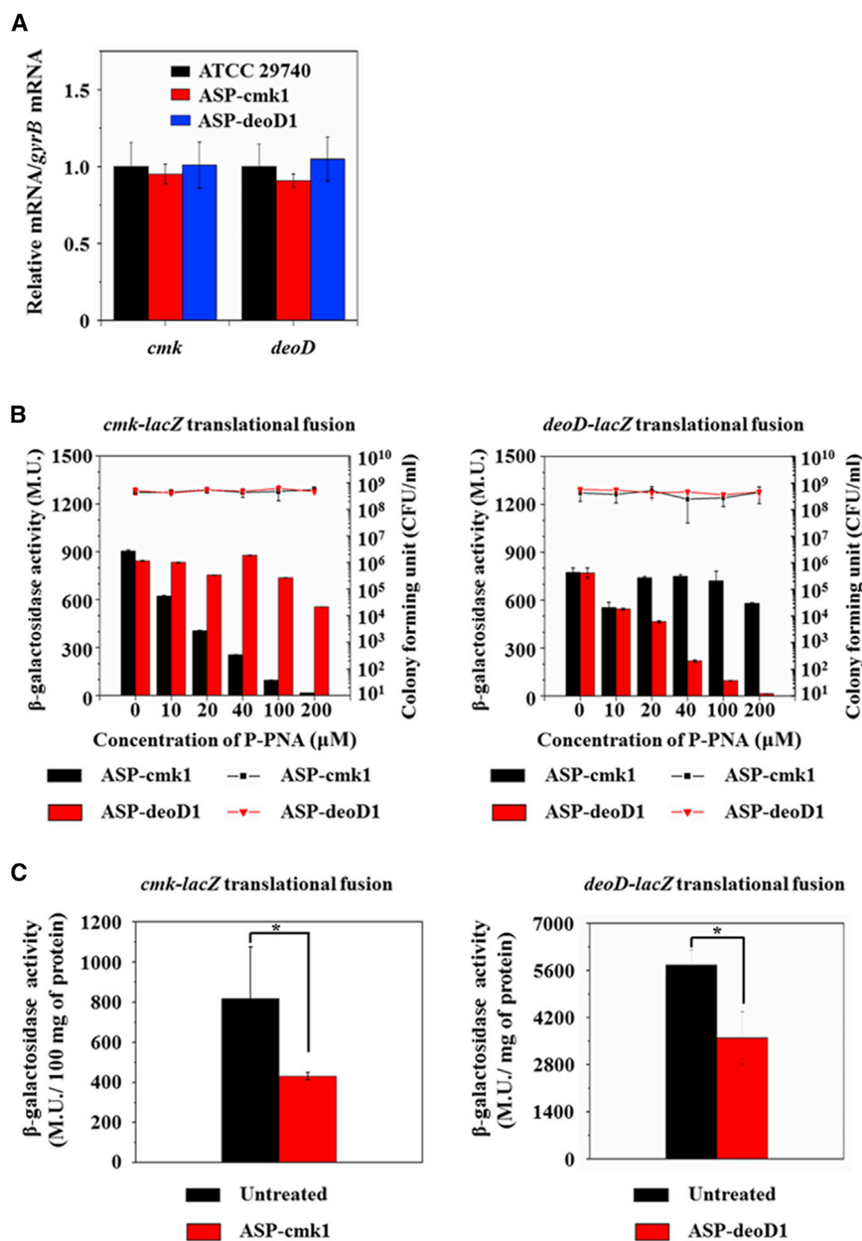

Figure 3. Antigene and Antisense Effects by ASP-cmk1 and ASP-deoD1

(A) The mRNA transcript levels of the *cmk* and *deoD* genes. *S. aureus* ATCC 29740 at approximately 1.0×10^9 CFU mL^{-1} was cultured for 4 h with 20 μM of each P-PNA. The relative expression levels of the *cmk* and *deoD* genes were analyzed using quantitative real-time PCR. The *gyrB* gene was used as a control. (B and C) β-galactosidase activity of the *lacZ* translational fusion constructs in *E. coli* (B) and *S. aureus* (C). The promoter regions of the *cmk* and *deoD* genes recognized by ASP-cmk1 and ASP-deoD1 were cloned to the promoter-less *lacZ* gene in pRS552 and either transformed to DH5α or further cloned in pMK4 for *S. aureus* transformation. The constructed reporter strains were cultured with either ASP-cmk1 or ASP-deoD1. β-galactosidase assays and BCA protein assays were performed as described in the Materials and Methods. Bacterial counts were measured using a standard direct plating method. The values are presented as the mean ± SD (n = 3). The asterisk indicates that the values are significantly different with a p value of < 0.05.

stringencies of individual genes.[21] For example, a slight decrease in the levels of mRNA transcripts of extremely important genes for survival can induce bacterial death, whereas that of genes that are less important cannot.[21] Therefore, we speculate that both *cmk* and *deoD* genes are extremely important because their complementary PNAs had the strongest inhibitory effects on the growth of *S. aureus*. Supporting this notion, the *cmk* and *deoD* genes encode cytidine monophosphate kinase and purine nucleoside phosphorylase, respectively,[20] which are involved in nucleotide biosynthesis, one of the most important metabolic pathways. Previous studies suggested that both genes are required for the growth of several bacterial species, such as *Corynebacterium glutamicum*, *Bacillus subtilis*, and *E. coli*,[22,23] and thus considered as potential therapeutic drug targets.[24]

As an alternative anti-infective strategy, various bactericidal P-PNAs have been developed against several single bacterial pathogens, including *E. coli*,[9,10] *S. aureus*,[11–15] *K. pneumoniae*,[16] and *P. aeruginosa*.[17] These PNA targets include genes involved in fatty acid or cell wall biosynthesis (e.g., *acpP*, *fabD*, *fabI*, *inhA*, *hmrB*, and *fmhB*), RNA polymerase sigma factors (e.g., *rpoD*), DNA replication (e.g., *gyrA*), or nucleotide precursor biosynthesis (e.g., *adk*).[17,25] However, *in vivo* anti-infectivity was demonstrated only with the *acpP*- or *rpoD*-targeting P-PNAs.[9,12] Although limited information is currently available on their effectiveness *in vivo*, the experimental evidence supports the idea that P-PNAs can effectively inhibit the growth of bacterial pathogens at micromolar concentrations without cellular toxicity, as well as without the induction of antibiotic resistance.[17]

Although none have reported on bactericidal P-PNAs with antigen activity, a few studies demonstrated the antibacterial activities by antisense P-PNAs, whose minimal inhibitory concentrations were similar to that of ASP-cmk1 in this study.[26] Moreover, a previous study revealed that antisense P-PNA could accumulate and retain in *E. coli*, which allowed a prolonged anti-bacterial effect.[27] Consistently, our *in vivo* applications of ASP-cmk1 demonstrated a clear reduction of bacterial loads in the intraperitoneally infected mice at

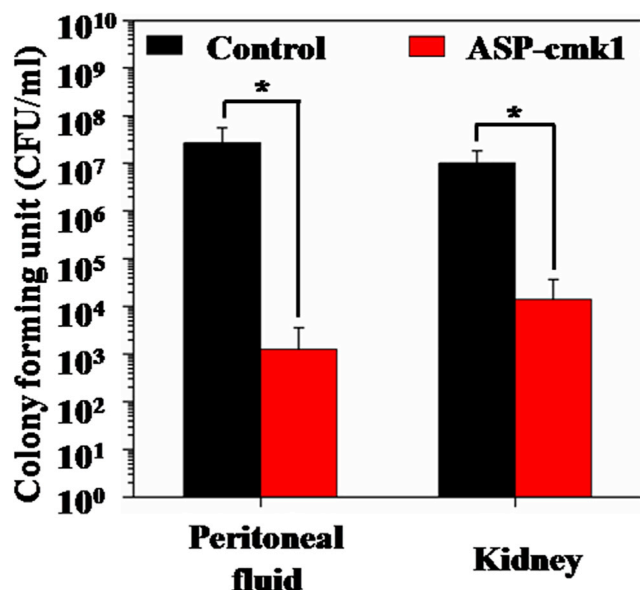

Figure 4. The *In Vivo* Effect of ASP-cmk1 Using a Mouse Intraperitoneal Infection Model

Mice were challenged intraperitoneally with *S. aureus* ATCC 29740 at approximately 2.0 × 10^8 CFU mL^{-1} in saline. After 30 min of bacterial challenge, the mice were injected with either saline (n = 5) or ASP-cmk1 (500 nmol; n = 5). At 20 h post-challenge, the bacterial loads in peritoneal fluid and kidney were measured using a standard direct plating method. The values are presented as the mean ± SD. The asterisk indicates that the values are significantly different with a p value of < 0.05.

20 h post-challenge ($p < 0.05$) as well as a significant increase in the survival rate of the breast-fed infant mice after intramammary infection of the lactating CD-1 mice over 72 h. Therefore, we speculate that ASP-cmk1 may have a strong bactericidal effect due to the sequence-specific antisense activity, not antigene, and induce a long post-antibiotic effect as previously described by Nikravesh et al.[27]

Interestingly, ASP-cmk1 appeared to have a broad spectrum of antibacterial activities, including Gram-negative *E. coli* O157:H7 and *Salmonella Typhimurium* DT104 (Figure S1). Although it has not been reported whether the $(KFF)_3K$ bacterial penetration peptide itself has any substantial antibacterial activities against certain bacterial species,[6,7,25] a recent study demonstrated that a partially mismatched antisense P-PNA exhibited considerable antibacterial activity, which was marginally lower than that of the fully matched P-PNAs.[12] It would be interesting to elucidate the possible mechanism(s) behind the observed broad-spectrum antibacterial activity of ASP-cmk1.

In conclusion, our data suggest that the P-PNA-mediated antisensing of essential genes commonly present in bacterial species provides a safe and effective tool to inhibit bacterial growth *in vivo* and thus can be useful as an alternative anti-infective strategy.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

S. aureus ATCC 29740 (American Type Culture Collection, Manassas, VA, USA),[18] MRSA N315,[19] and three Korean bovine mastitis isolates of *S. aureus* were maintained on either Luria-Bertani (LB; BD Difco, Sparks, MD, USA) or 5% sheep blood agar (KOMED, South Korea) plates at 37°C. Bacterial stocks were prepared with fresh LB media containing 40% (v/v) glycerol and kept at −80°C until use. The reagents were used at the following concentration: ampicillin (AMP), 200 μg mL^{-1}; kanamycin (KAN), 50 μg mL^{-1}; 5-bromo-4-chloro-3-indoyle-β-D-galactopyranoside (X-gal), 20 μg mL^{-1}.

Design and Synthesis of P-PNAs

A total of 52 potentially essential genes conserved in various bacterial species including *S. aureus*, *Escherichia coli*, *Streptococcus pneumoniae*, *Francisella novicida*, *Acinetobacter baylyi*, *Pseudomonas aeruginosa*, and *Vibrio cholera* were previously identified by Duffield et al.[20] from the Database of Essential Genomes (http://www.essentialgene.org/), according to the down-selection method. Among them, seven genes (*cmk*, *deoD*, *ligA*, *smpB*, *glmU*, *pyrH*, and *ftsA*) were experimentally validated as being essential at least in *Yersinia pseudotuberculosis* YPIII by checking the lethality of targets.[20] To evaluate the essentiality of these genes in *S. aureus* as a model organism, the gene-specific oligonucleotides were searched in the genome sequence of MRSA N315 (GenBank: BA000018), and the resulting PNA oligomers for the individual target genes were designed to bind their translation initiation regions within the coding strands of each mRNA,[10,11] which overlapped the AUG start codon and/or the ribosome-binding Shine-Dalgarno sequences (Table 1). All the PNAs were synthesized, purified, and conjugated with the $(KFF)_3K$-L bacterial penetration peptide at PANAGENE (Daejeon, South Korea) for efficient delivery into bacterial cells (Table 1).

Standard Spot Assay

Bacteria were precultured in Mueller-Hinton (MH; BD Difco) broth at 37°C for 18 h and diluted 1:1,000 with fresh MH broth to obtain a bacterial concentration of 6.0 ± 1.6 × 10^4 colony forming units (CFU) mL^{-1}. The indicated concentrations of P-PNAs were then added to the bacterial diluents and further incubated at 37°C without aeration. After incubation for 4 to 8 h, bacterial cultures were put on ice to stop the growth and serially diluted with fresh MH broth using sterile Eppendorf tubes. For the spot assay, 5 μL of each bacterial diluent was dropped onto MH agar plates, allowed to be absorbed, and incubated at 37°C for 24 h.

Determination of MECs of P-PNAs

S. aureus ATCC 29740 at approximately 5.0 × 10^4 CFU mL^{-1} was cultured in MH broth at 37°C with 0, 5, 10, 20, 40 μM of the following P-PNAs: ASP-cmk1 and ASP-deoD1. After 4 h of incubation, the bacterial cultures were serially diluted and spotted onto MH agar plates as described above. The plates were then incubated at 37°C for 24 h and the viable cells were counted. The data are represented as the means ± SD CFU mL^{-1} from at least three independent experiments.

Table 2. The *In Vivo* Effect of ASP-cmk1 in Infant Mice Survival against Intramammary *S. aureus* Infection

Infection Dosage (CFU/Mouse)	Treatment	Number of Suckling Infant Mice	Number of Surviving Mice at 24 h after Infection (n)[a]
1.4×10^2	saline	20	0
	ASP-cmk1[b]	25	25
4.0×10^2	saline	19	0
	ASP-cmk1[b]	22	22
2.1×10^3	saline	21	0
	ASP-cmk1[b]	21	21

[a]The number of surviving mice was monitored up to 72 h after infection; no changes were observed.
[b]The lactating mice were injected with 500 nmol ASP-cmk1 at 30 min after bacterial challenge.

Quantitative Real-Time PCR

S. aureus ATCC 29740 at approximately 1.0×10^9 CFU mL^{-1} was cultured in MH broth at 37°C with 20 μM of the following P-PNAs: ASP-cmk1 and ASP-deoD1. After 4 h of incubation, the total bacterial RNAs were extracted using RNeasy mini kit (QIAGEN, Hilden, Germany). The resultant RNAs were treated with RNase-free DNase I (Ambion, Austin, TX, USA) to eliminate all contaminating DNAs. The total RNAs were reverse-transcribed into cDNA using PrimeScript Reverse Transcriptase (Takara, Shiga, Japan), and the quantitative real-time PCR was performed using LightCycler 96 (Roche Diagnostics, Mannheim, Germany) and FastStart Essential DNA Green Master (Roche Diagnostics). The thermal conditions are as follows: a preincubation at 95°C for 10 min, followed by 45 cycles with denaturing at 95°C for 10 s, annealing at 60°C for 10 s, and elongation at 72°C for 10 s. The *gyrB* gene was used as a control. All the primers in this study are listed in Table 3. The gene expression levels were analyzed using the comparative delta-delta Ct ($2^{-\Delta\Delta Ct}$) method.[28]

Construction of a Multicopy *lacZ* Translational Fusion

The translational fusion vector pRS552 carrying a promoter-less *lacZ* gene[29] was used to analyze translational activity of the *cmk* and *deoD* genes. The promoter regions (approximately 400 bp) of the *cmk* and *deoD* genes recognized by ASP-cmk1 and ASP-deoD1 were amplified from the genomic DNA of *S. aureus* ATCC 29740 using the gene-specific primers (Table 3). The PCR products were digested with BamHI and EcoRI (New England Biolabs, Ipswich, MA, USA) and ligated into the corresponding restriction sites of pRS552. After transformation into *E. coli* DH5α by a heat-shock method, the transformants were screened for ampicillin and kanamycin resistance (AMP^R/KAN^R) and blue or white phenotype. The putative inserts in candidate transformants were confirmed by DNA nucleotide sequencing (COSMO GENETECH, Seoul, South Korea).

A shuttle vector with the desired promoter-*lacZ* fragment was constructed as follows: the aforementioned translational fusion vectors were used as templates for amplifying *cmk*/*deoD-lacZ* fusion fragments; the desired DNA regions were amplified using Q5 high-fidelity DNA polymerase (New England Biolabs) and primers lacZ-cmk-F/lacZ-deoD-F and pRS552lacZ-R. An *E. coli-Bacillus* shuttle vector pMK4[30] and the amplified fragments were digested using EcoRI and SalI (New England Biolabs), ligated, and transformed to *E. coli* DH5α. Insertion of the fusion fragment in pMK4 was confirmed by restriction enzyme digestion and DNA sequencing. The constructed vectors, pMKcmk and pMKdeoD, were subcloned into *S. aureus* RN4220 by electroporation. Expression of β-galactosidase in transformed RN4220 was confirmed by streaking on an LB agar plate treated with X-gal.

β-Galactosidase Assay

For *E. coli* β-galactosidase assay, the translational fusion constructs were grown overnight at 37°C in LB broth with agitation at 250 rpm and diluted 1:100 in LB broth containing AMP with various concentrations of ASP-cmk1 or ASP-deoD1. After 4 h of culture at 37°C and 250 rpm, the β-galactosidase activity was measured with 0.1% SDS and chloroform permeabilized cells as previously described.[31] Bacterial counts were measured using a standard direct plating method. At least three independent experiments were performed.

For *S. aureus* β-galactosidase assay, RN4220 strains harboring the translational fusion constructs were grown overnight at 37°C in LB broth with agitation (250 rpm) and diluted 1:100 in fresh MH broth containing chloramphenicol (30 μg/mL). ASP-cmk1 or ASP-deoD1 was then added to the culture to a final concentration of 20 μM and further incubated for 4 h. Cells were collected by centrifugation (20,000 × *g*, 4°C, 3 min) and resuspended in 500 μL Z buffer (40 mM Na_2HPO_4, 60 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol [pH 7]) with 0.5 mg/mL DNase I, 5 mM DTT, and 0.1 mg/mL lysostaphin. Samples were incubated at 37°C for 30 min to lyse cells, and cell debris were removed by centrifugation (20,000 × *g*, 4°C, 3 min). Supernatants containing lysates were either used directly for assays or stored at −20°C until further use. Protein concentrations were determined using Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

β-galactosidase activity was measured using the method described previously[31] with modifications. In brief, 10 μL of the protein sample was mixed with 90 μL Z buffer and 20 μL *o*-nitrophenyl-β-D-galactopyranoside (ONPG) in 96-well plate. The reaction was stopped by adding 50 μL 1 M Na_2CO_3 when the mixture turned straw yellow. Absorbance at 420 nm was measured using the Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA). β-galactosidase-specific activity was expressed as Miller units mg^{-1} protein. All data were from three independent experiments.

In Vivo Application of ASP-cmk1 Using Murine Infection Models

All the experimental and animal care procedures were approved by Seoul National University Institutional Animal Care and Use Committees (SNU-121025-1) and performed in compliance with the standard guidelines. A power analysis (GPower3.1.9.3; a = 0.05,

Table 3. Primer Oligonucleotide Sequences in This Study

Primer Name	Oligonucleotide Sequence (5′ to 3′)	Reference
For Quantitative Real-Time PCR		
cmk-F	GCATCAGTTGAAGAGCGAGC	this study
cmk-R	TCGACTTGCCTGTCGTATCT	this study
deoD-F	CGGTTCTTGTGGCGCATTAC	this study
deoD-R	ACTCGAAGTCAGCGATAGGC	this study
gyrB-F	TAGTCGAAGGGGACTCTGCC	this study
gyrB-R	TCAAAGTCGCCACCGATTCC	this study
For Translational Fusion Constructs		
lacZ-cmK-F	GGGAATTCCGTATCGGTACCATGCGTGA (EcoRI linker)	this study
lacZ-cmK-R	CGGGATCCGCAGCAGCTGGACCATCTAA (BamHI linker)	this study
lacZ-deoD-F	CAGAATTCCGTACGTGTTGACTCAGGCT (EcoRI linker)	this study
lacZ-deoD-R	CAGGATCCGGTTGAATATGTGGTGTACCT (BamHI linker)	this study

power = 0.90; effect size calculation based on estimated effect sizes in surviving infant mice of ASP-cmk1 and saline group) was used to determine appropriate number of animals.

Six-week-old female C57BL/6J mice, weighing 18 to 22 g, were purchased from Central Lab Animal (Seoul, South Korea). *S. aureus* ATCC 29740 at approximately 2.0 × 10^8 CFU was prepared in 100 μL of saline. After being acclimated for 3 days, mice were challenged intraperitoneally with *S. aureus* using a 26G needle syringe. After 30 min of bacterial challenge, the mice were injected with either sterile saline (n = 5) or ASP-cmk1 (500 nmol per mouse; n = 5). It is noteworthy that the concentration of ASP-cmk1 was decided by considering the average body weight (∼20 g) of mice and the MEC of ASP-cmk1 previously determined *in vitro* (see Results and Figure 2). At 20 h post-challenge, the mice were euthanized and a peritoneal flush was performed by injecting 2 mL of sterile saline to observe bacterial counts. Bacterial loads in peritoneal fluid and kidney were measured using a standard direct plating method.

The *in vivo* effect of ASP-cmk1 in a murine intramammary infection model was investigated as previously described with slight modifications.[32] Ten pregnant CD-1 mice were purchased from Central Lab Animal (Seoul, South Korea). At 12–14 days after birth, all the infant mice were moved to new cages temporally before intramammary infection of the CD-1 lactating mice. In brief, anesthesia was performed on the lactating mice (n = 5 per group) with a mixture of ketamine and xylazineas previously described.[32] *S. aureus* ATCC 29740 at approximately 1.4 × 10^2 to 2.1 × 10^3 CFU were prepared in 100 μL of saline. Using a 33G blunt needle syringe, bacteria were inoculated into the L4 and R4 abdominal mammary glands of the mice. At 30 min after bacterial challenge, the lactating mice were injected with either sterile saline (n = 5, the control group) or ASP-cmk1 (500 nmol per mouse; n = 5, the P-PNA-treated group) as above. All the suckling infant mice (n = 19 to 25 per group) were then returned for breast-feeding, and their survival rates were determined at 12, 24, 48, and 72 h post-inoculation.

In Vivo Toxicity of ASP-cmk1 in Mice

Female CD-1 mice were purchased from Central Lab Animal (Seoul, South Korea). After being acclimated for 3 days, mice were injected intravenously with either sterile saline (n = 10) or ASK-cmk1 (1,000 nmol; n = 10). Body weight, food and water consumption, and mice behavioral activities were observed for 10 days post-administration. At the 10th day, mice were euthanized and organs (liver, lungs, mammary gland, and intestine) were collected. Histopathological analysis of the extracted organs was performed after H&E staining.

Statistical Analysis

Student's t test and ANOVA were used to evaluate statistical significance using the SPSS software program (SPSS ver. 12; SPSS, Chicago, IL, USA). A p value of < 0.05 was considered to indicate statistical significance in this study.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.08.021.

AUTHOR CONTRIBUTIONS

J.W.Y. conceived and designed experiments and wrote the paper; H.T.L. and S.K.K. were involved with all experiments and wrote the paper; J.B.L. helped with *S. aureus* genetic experiments.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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