Published in final edited form as:

Int J Antimicrob Agents. 2013 April ; 41(4): . doi:10.1016/j.ijantimicag.2012.11.017.

Peptide nucleic acids inhibit growth of *Brucella suis* in pure culture and in infected murine macrophages

Parthiban Rajasekaran^a, Jeffry C. Alexander^a, Mohamed N. Seleem^{b,1}, Neeta Jain^a, Nammalwar Sriranganathan^a, Alice R. Wattam^c, João C. Setubal^{c,d}, and Stephen M. Boyle^{a,*}

^aCenter for Molecular Medicine and Infectious Diseases, Virginia-Maryland College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

^bInstitute for Critical Technology and Applied Science, Blacksburg, VA 24061, USA

^cVirginia Bioinformatics Institute, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

^dDepartment of Biochemistry, Institute of Chemistry, University of São Paulo, São Paulo, SP 05508, Brazil

Abstract

Peptide nucleic acids (PNAs) are single-stranded, synthetic nucleic acid analogues containing a pseudopeptide backbone in place of the phosphodiester sugar-phosphate. When PNAs are covalently linked to cell-penetrating peptides (CPPs) they readily penetrate the bacterial cell envelope, inhibit expression of targeted genes and cause growth inhibition both of Gram-positive and Gram-negative bacteria. However, the effectiveness of PNAs against *Brucella*, a facultative intracellular bacterial pathogen, was unknown. The susceptibility of a virulent *Brucella suis* strain to a variety of PNAs was assessed in pure culture as well as in murine macrophages. The studies showed that some of the PNAs targeted to *Brucella* genes involved in DNA (*polA*, *dnaG*, *gyrA*), RNA (*rpoB*), cell envelope (*asd*), fatty acid (*kdtA*, *acpP*) and protein (*tsf*) synthesis inhibit the growth of *B. suis* in culture and in macrophages after 24 h of treatment. PNA treatment inhibited *Brucella* growth by interfering with gene expression in a sequence-specific and dose-dependent manner at micromolar concentrations. The most effective PNA in broth culture was that targeting *polA* at ca. 12 μ M. In contrast, in *B. suis*-infected macrophages, the most effective PNAs were those targeting *asd* and *dnaG* at 30 μ M; both of these PNAs had little inhibitory effect on *Brucella* in broth culture. The *polA* PNA that inhibits wild-type *B. suis* also inhibits the growth of wild-type *Brucella melitensis* 16M and *Brucella abortus* 2308 in culture. This study reveals the potential usefulness of antisense PNA constructs as novel therapeutic agents against intracellular *Brucella*.

© 2012 Elsevier B.V. and the International Society of Chemotherapy. All rights reserved.

*Corresponding author. Present address: 1410 Price's Fork Road, Blacksburg, VA 24061, USA. Tel.: +1 540 231 4641; fax: +1 540 231 3426., smbryle@vt.edu (S.M. Boyle).

¹Present address: College of Veterinary Medicine, Purdue University, West Lafayette, IN 47907, USA.

Competing interests: None declared.

Ethical approval: Not required.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Peptide nucleic acid (PNA); *Brucella*; Antibiotic resistance; Antisense; Murine macrophage

1. Introduction

Targeting bacterial genes using antisense technologies has recently received attention in Gram-positive, Gram-negative and acid-fast bacteria [1]. Many of the studies reveal significant impairment of bacterial growth as a function of treatment with specific antisense constructs. New nucleic acid analogues and mimics such as peptide nucleic acids (PNAs) have been developed and are found to form exceptionally strong complexes with complementary strands of DNA and/or mRNA, which leads to inhibition of gene expression as well as inhibition of bacterial growth [2–4].

Human brucellosis is one of the five most common bacterial zoonoses in the world; over 500 000 new cases are estimated by the World Health Organization (WHO) to occur annually [5]. Human brucellosis patients are treated with combinations of antimicrobials such as rifampicin and doxycycline or streptomycin and doxycycline for 6–8 weeks. In general, 2% of untreated patients infected with *Brucella melitensis* die and the relapse rate ranges from 5% to 15% (<http://www.bt.cdc.gov/agent/brucellosis/>). The Brucellae are resistant to a number of commonly used antimicrobials [6], and novel therapeutic agents are needed to treat brucellosis more efficiently. Moreover, in its chronic phase, successful antimicrobial therapy is difficult since *Brucella* spp. are intracellular pathogens (i.e. replicate inside of macrophages), which puts them out of ready access of many bactericidal agents as well as specific antibodies [7,8]. As such, newer approaches are needed to ensure that the antimicrobial or therapeutic molecules reach inside the macrophages, the intracellular niche of *Brucella*.

As a first step in developing a novel therapeutic approach for the treatment of brucellosis, the susceptibility of *Brucella suis* growth to a variety of PNAs was tested both in pure culture and in infected murine macrophages. The studies here show that PNAs targeted to *Brucella* genes encoding enzymes involved in DNA, RNA, cell envelope and protein synthesis can inhibit the growth of *B. suis* both in culture and in infected macrophages.

2. Materials and methods

PNA (Table 1) were synthesised by Panagene Inc. (Daejeon, South Korea). PNAs were conjugated with the cell-penetrating peptide (CPP) KFFKFFKFFK [9] to facilitate their uptake through the bacterial cell envelope [10].

2.1. Bacterial strains

Brucella suis 1330, *B. melitensis* 16M and *Brucella abortus* 2308 are wild-type strains obtained from the culture collection of the Virginia-Maryland Regional College of Veterinary Medicine at Virginia Tech (Blacksburg, VA) and were grown in tryptic soy broth (TSB) (Difco, Franklin Lakes, NJ) or on tryptic soy agar (TSA) (Difco) at 37 °C in 5% CO₂. All growth experiments with *Brucella* spp. were performed in a BSL-3 laboratory located at the Virginia-Maryland Regional College of Veterinary Medicine at Virginia Tech (Blacksburg, VA) and certified by the US Centers for Disease Control and Prevention (CDC).

2.2. Brucella growth in pure culture

Determination of the susceptibility of *Brucella* to PNAs was carried out as a growth inhibitory concentration assay because the cost of PNAs (ca. \$800 per 40 μ mol) precluded routine testing in a traditional two-fold dilution series. Still in this non-conventional format, the effective growth inhibitory concentration is defined as the lowest concentration of PNA required for a statistically significant reduction in the viable bacterial count after 24 h or 48 h of incubation compared with the control. Since the expression of essential genes is inhibited, inhibition is considered bactericidal. A frozen stock of *B. suis* was diluted in TSB to 1.0×10^4 CFU/mL and was incubated with indicated concentrations of PNA (volume = 30 μ L) in triplicate using a 96-well (total volume = 100 μ L/well), low adhesion, microtitre plate (cat. # 3474; Corning Inc., Corning, NY). The plate was sealed with an adhesive lid (Microseal[®] B; Bio-Rad, Hercules, CA) and was incubated at 37 °C in a VersaMax[™] Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA) at the following settings: kinetic, 550 nm; before readings, plates were shaken for 10 s, read every 15 min, reduction set at 4. To reduce edge effects and evaporation, the wells on the entire perimeter of the plate were not used for growth but had 200 μ L of TSB added to help reduce evaporation. Incubation of the plate containing PNA-treated cultures in a microplate reader allowed for monitoring differences in optical density at 550 nm (OD₅₅₀) as a function of time compared with the untreated control as described elsewhere [9,11]. At various time points post treatment as mentioned for each experiment, the PNA-treated *B. suis* was serially diluted and plated on TSA plates. The plates were incubated at 37 °C under 5% CO₂ for 3 days and CFU were estimated by colony counting and correcting for dilution to estimate CFU/mL.

2.3. Inhibition of Brucella growth in a macrophage cell line

Murine macrophage-like cells J774A.1 (ATCC, Manassas, VA) were seeded at a density of ca. 2×10^4 cells/well in 96-well plates (Corning Inc.) 24 h prior to infection. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St Louis, MO) and were infected (multiplicity of infection = 100) with *B. suis* as described previously [12]. At 24 h post infection, the cells were washed two times with 200 μ L of DMEM, re-suspended in 100 μ L of DMEM with PNAs (30 μ M) and 10% fetal calf serum (FCS) and then incubated for a further 24 h. Infected macrophages were washed three times with phosphate-buffered saline (PBS) and were lysed with 0.1% Triton-X 100 (Sigma-Aldrich). CFU of *B. suis* in the lysates were determined by plating 10-fold serial dilutions onto TSA and incubating the plates at 37 °C under 5% CO₂.

2.4. Macrophage viability and toxicity assays

To determine whether PNA containing CPP or CPP alone had an undesirable effect on macrophages, a tetrazolium compound-based cell viability assay [CellTiter 96[®] Aqueous Non-Radioactive Cell Proliferation Assay (MTS assay); Promega, Fitchburg, WI] was performed on J774A.1 macrophages. The macrophages were incubated with 30 μ M of either PNA with CPP or CPP alone for 24 h at 37 °C under 5% CO₂. The number of viable cells was measured based on the amount of absorbance at 490 nm due to formazan product. Cell viability was also measured following 24 h of PNA treatment by trypan blue exclusion assay and counting the number of viable cells using a gridded haemocytometer; 100 cells were counted to determine the percentage of live cells.

2.5. Statistical analysis

All statistical analyses were performed with Student's two-tailed *t*-test using Microsoft Excel (Microsoft Corp., Redmond, WA). *P*-values of 0.05 were considered significant.

3. Results

To test the antibacterial activity of PNAs targeted to genes involved in different types of macromolecular synthesis (DNA, RNA, lipid, cell envelope, protein), a number of PNAs complementary to the start codon region encoded by selected genes in *B. suis* were evaluated (Table 1).

The targets in this table were identified and selected as they are considered essential genes for both the functioning and growth of *Brucella* using the bioinformatics resources and tools at PathoSystems Resource Information Center (PATRIC; <http://www.cs.vt.edu/node/632>) (<http://patricbrc.vbi.vt.edu/portal/portal/patric/IncumbentBRCs?page=eric>). The results of several PNA inhibition experiments on *B. suis* cultured in microtitre plates are summarised in Table 2. Results show that four of the eight specific PNAs significantly inhibited the growth of *B. suis* in culture: the four PNAs targeted genes encoded by *kdtA* (a transferase affecting lipid A), *tsf* (elongation factor Ts), *polA* (DNA polymerase I) and *rpoB* (α-subunit of RNA polymerase).

In each of these constructs, CPP represents approximately one-quarter of the conjugate mass (ca. 8 μM). In addition, the negative controls (water or anti-*lacZ* PNA conjugated with CPP at 30 μM) did not inhibit the growth of *B. suis*. Moreover, neither the CPP (20 μM) by itself (Table 3) nor any of the PNAs not attached to the CPP (data not shown) were capable of inhibiting *B. suis* in culture.

To determine the minimum growth inhibitory concentration of *polA* PNA, *B. suis* was incubated with a range of concentrations for 72 h in TSB. From the results in Table 4, it is clear that the minimum growth inhibitory concentration is ca. 12.5 μM. Moreover, these results were validated when the CFU of *B. suis* were shown to be reduced by 9 log reduction at 20 μM *polA* PNA compared with untreated *B. suis* (Table 4).

Because *Brucella* infect and replicate inside a variety of phagocytic cells, including macrophages, it was important to know whether the PNAs would inhibit growth once *Brucella* are in that intracellular location. To be effective, the PNAs would have to penetrate the cell membranes associated with both the macrophage and the endosomal compartment, termed a replicative phagosome or replisome, where *Brucella* reside and replicate [13]. Because the mouse is used as a model to study brucellosis, the ability of the PNAs to inhibit growth of intracellular *B. suis* within the infected murine macrophage cell line J774.A1 was determined. The results in Table 5 show the ability of PNAs to inhibit growth of intracellular *B. suis*. Compared with the negative controls, those PNAs targeted to *asd*, *gyrA*, *dnaG* and *polA* showed significant inhibition of *B. suis* after 24 h. To determine whether PNAs linked to CPP, PNAs unlinked to CPP, or the CPP by itself was having any toxic effects on the J774.A1 macrophages and thereby indirectly affecting intracellular *B. suis* survival, both the viability and the reductive capacity (based on mitochondria activity and cell metabolism) of the cell line were measured after 24 h of PNA treatment. There was no detectable toxic effect noticed either in a trypan blue exclusion assay or in the cell viability MTS assay even at the highest concentration of 30 μM of each PNA derivative (data not shown). In addition, the PNAs did not visibly alter J774.A1 cell growth in terms of morphology or doubling time at the concentrations tested (data not shown).

4. Discussion

The ability of PNAs designed to target specific genes involved in the synthesis of DNA, RNA, proteins, fatty acids and the cell wall in *B. suis* suggests a greatly expanded opportunity to control growth of this pathogen. Essentially, in the case of the average bacterium containing 3000–4000 genes (ca. 3300 in *B. suis* [14]), each essential gene could

be a potential target for limiting growth of the species. It is noteworthy that not all of the PNAs that were effective in limiting *Brucella* growth in macrophages were effective in pure cultures (see Tables 2 and 5). In the case of PNAs designed to inhibit expression of the *asd* gene, which encodes aspartate-semialdehyde dehydrogenase that synthesises diaminopimelic acid necessary for cross-linking of peptidoglycan layers, and of the *gyrA* and *dnaG* genes encoding enzymes involved in DNA replication, all of them had significant growth inhibitory effects on *Brucella* in macrophages but not in *Brucella* cultures. In contrast, the PNA designed to inhibit expression of *polA* encoding DNA polymerase I necessary for DNA replication, appears to be effective both in pure culture and in infected macrophages in vitro. At this point, we can speculate that intracellular *Brucella* possess a different set of conditionally essential genes compared with the ones growing in TSB culture medium. It has been noted that the intracellular environment of a macrophage is not enriched with substrates that facilitate bacterial metabolism, e.g. amino acids, such that bacterial auxotrophs have impaired growth in the macrophage [15]. Earlier work in our laboratory has shown that a leucine auxotroph of vaccine strain *B. abortus* RB51 can overexpress antigens as efficiently as the parent strain with the aid of a plasmid-borne complementation system that utilises the intracellular nutritive environment to its advantage [16,17]. In the case of *B. suis*, amino acid as well as purine and pyrimidine auxotrophs are attenuated in macrophages, which is consistent with a lack of these precursors in this intracellular environment [18]. We would expect PNAs that inhibit genes involved in the synthesis of metabolites that can be taken up from the medium to be much more effective in minimal medium compared with an enriched medium. We have not assessed the growth inhibitory properties of PNAs against *Brucella* in a minimal medium.

The concentrations of PNAs required to inhibit *B. suis* growth in culture and in macrophages (12–30 μM) are in line with those reported for other bacterial species: *Staphylococcus aureus*, 4–10 μM [9] and 12–40 μM [19]; *Escherichia coli*, 6–12 μM [19,20]; *Mycobacterium smegmatis*, 5–15 μM [21]; and *Klebsiella pneumoniae*, 10–40 μM [22]. In the case of human epithelial cells infected with *K. pneumoniae*, 20–40 μM PNAs specific for *ompA* or *gyrA* eliminated the bacterial pathogen following overnight incubation [22].

Furthermore, the *polA* PNA was effective against two closely related *Brucella* spp., namely *B. melitensis* 16M and *B. abortus* 2308 (data not shown). These latter results are not unexpected given that the target sequences of the *polA* (11 nucleotides defined in Table 1) are identical amongst these three species that cause the majority of infections in animals and humans. Also, we were able to confirm (data not shown) the lack of CPP-mediated toxicity to eukaryotic cells with two different assays (MTS and trypan blue exclusion assays) as has been observed by others [10,23].

The observation that the PNAs are effective in limiting the growth of *Brucella* inside macrophages (Table 5) opens the possibility of treating brucellosis in vertebrates. The choice of PNAs for macrophage experiments was made by taking into account the PNAs that inhibited broth cultures and the putative function of the target gene in the intracellular compartment. Delivery of therapeutic PNAs to the intracellular niche of bacterial residence and replication might allow an explanation for possible differences in effectiveness of PNAs between broth culture and macrophage infection. None the less, there is a clear need for the development of a carrier system that will deliver the PNAs specifically to *Brucella*-infected tissues in order to reduce the therapeutic dose and to avoid off-target effects. In the case of brucellosis, this scenario is somewhat simplified by the fact that *Brucella* is an intracellular pathogen and spends a lot of its life inside phagocytic cells such as macrophages. Thus, delivery systems that take advantage of receptor–ligand interactions associated with the macrophage have a higher probability of success in allowing the PNAs to deliver a therapeutic effect, as the CPP associated with the PNA is non-discriminatory with respect to

the type of cell membrane it penetrates [10]; both mammalian and microbial membranes as well as bacterial cell envelopes are not major barriers. Because there are numerous genes making up the bacterial genome, the potential for novel DNA-based antimicrobials like PNAs has increased considerably. What remains to be demonstrated is whether the PNAs can alter the course of a *Brucella* infection. Studies are currently underway to assess the use of various forms of targeted nanoparticles, including liposomes, to deliver PNAs to animal tissues infected with *Brucella*.

Acknowledgments

The authors thank Liam Good of the Royal Veterinary College at the University of London (London, UK) for providing expertise on peptide nucleic acids as well as commentary on the manuscript.

Funding: This research was supported by grants from the Virginia-Maryland Regional College of Veterinary Medicine (Blacksburg, VA) to NS and SMB and by the National Institutes of Health (NIH) (5R03AI083735-02) to SMB. Bioinformatics work involving ARW was funded in whole or in part by federal funds from the National Institute of Allergy and Infectious Diseases, NIH, Department of Health and Human Services, under contract no. HHSN272200900040C, awarded to B.W. Sobral.

References

1. Rasmussen LC, Sperling-Petersen HU, Mortensen KK. Hitting bacteria at the heart of the central dogma: sequence-specific inhibition. *Microb Cell Fact*. 2007; 6:24. [PubMed: 17692125]
2. Good L, Nielsen PE. Antisense inhibition of gene expression in bacteria by PNA targeted to mRNA. *Nat Biotechnol*. 1998; 16:355–8. [PubMed: 9555726]
3. Good L, Nielsen PE. Inhibition of translation and bacterial growth by peptide nucleic acid targeted to ribosomal RNA. *Proc Natl Acad Sci U S A*. 1998; 95:2073–6. [PubMed: 9482840]
4. Good L, Nielsen PE. Peptide nucleic acid (PNA) antisense effects in *Escherichia coli*. *Curr Issues Mol Biol*. 1999; 1:111–6. [PubMed: 11475695]
5. Seleem MN, Boyle SM, Sriranganathan N. Brucellosis: a re-emerging zoonosis. *Vet Microbiol*. 2010; 140:392–8. [PubMed: 19604656]
6. Halling SM, Jensen AE. Intrinsic and selected resistance to antibiotics binding the ribosome: analyses of *Brucella* 23S rrn, L4, L22, EF-Tu1, EF-Tu2, efflux and phylogenetic implications. *BMC Microbiol*. 2006; 6:84. [PubMed: 17014718]
7. Conde-Álvarez R, Arce-Gorvel V, Iriarte M, Manek-Keber M, Barquero-Calvo E, Palacios-Chaves L, et al. The lipopolysaccharide core of *Brucella abortus* acts as a shield against innate immunity recognition. *PLoS Pathog*. 2012; 8:e1002675. [PubMed: 22589715]
8. Pizarro-Cerdá J, Méresse S, Parton RG, van der Goot G, Sola-Landa A, Lopez-Goñi I, et al. *Brucella abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. *Infect Immun*. 1998; 66:5711–24. [PubMed: 9826346]
9. Nekhotiaeva N, Awasthi SK, Nielsen PE, Good L. Inhibition of *Staphylococcus aureus* gene expression and growth using antisense peptide nucleic acids. *Mol Ther*. 2004; 10:652–9. [PubMed: 15451449]
10. Fonseca SB, Pereira MP, Kelley SO. Recent advances in the use of cell-penetrating peptides for medical and biological applications. *Adv Drug Deliv Rev*. 2009; 61:953–64. [PubMed: 19538995]
11. Ghosal A, Nielsen PE. Potent antibacterial antisense peptide–peptide nucleic acid conjugates against *Pseudomonas aeruginosa*. *Nucleic Acid Ther*. 2012; 22:323–34. [PubMed: 23030590]
12. Seleem MN, Jain N, Pothayee N, Ranjan A, Riffle JS, Sriranganathan N. Targeting *Brucella melitensis* with polymeric nanoparticles containing streptomycin and doxycycline. *FEMS Microbiol Lett*. 2009; 294:24–31. [PubMed: 19493005]
13. Bellaire BH, Roop RM 2nd, Cardelli JA. Opsonized virulent *Brucella abortus* replicates within nonacidic, endoplasmic reticulum-negative, LAMP-1-positive phagosomes in human monocytes. *Infect Immun*. 2005; 73:3702–13. [PubMed: 15908400]

14. Paulsen IT, Seshadri R, Nelson KE, Eisen JA, Heidelberg JF, Read TD, et al. The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. *Proc Natl Acad Sci U S A*. 2002; 99:13148–53. [PubMed: 12271122]
15. Appelberg R. Macrophage nutriptive antimicrobial mechanisms. *J Leukoc Biol*. 2006; 79:1117–28. [PubMed: 16603587]
16. Rajasekaran P, Seleem MN, Contreras A, Purwantini E, Schurig GG, Sriranganathan N, et al. *Brucella abortus* strain RB51 leucine auxotroph as an environmentally safe vaccine for plasmid maintenance and antigen overexpression. *Appl Environ Microbiol*. 2008; 74:7051–5. [PubMed: 18836016]
17. Rajasekaran P, Surendran N, Seleem MN, Sriranganathan N, Schurig GG, Boyle SM. Over-expression of homologous antigens in a leucine auxotroph of *Brucella abortus* strain RB51 protects mice against a virulent *B. suis* challenge. *Vaccine*. 2011; 29:3106–10. [PubMed: 21376799]
18. Kohler S, Foulongne V, Ouahrani-Bettache S, Bourg G, Teyssier J, Ramuz M, et al. The analysis of the intramacrophagic virulome of *Brucella suis* deciphers the environment encountered by the pathogen inside the macrophage host cell. *Proc Natl Acad Sci U S A*. 2002; 99:15711–6. [PubMed: 12438693]
19. Bai H, You Y, Yan H, Meng J, Xue X, Hou Z, et al. Antisense inhibition of gene expression and growth in Gram-negative bacteria by cell-penetrating peptide conjugates of peptide nucleic acids targeted to *rpoD* gene. *Biomaterials*. 2012; 33:659–67. [PubMed: 22000398]
20. Dryselius R, Aswasti SK, Rajarao GK, Nielsen PE, Good L. The translation start codon region is sensitive to antisense PNA inhibition in *Escherichia coli*. *Oligonucleotides*. 2003; 13:427–33. [PubMed: 15025910]
21. Kulyte A, Nekhotiaeva N, Awasthi SK, Good L. Inhibition of *Mycobacterium smegmatis* gene expression and growth using antisense peptide nucleic acids. *J Mol Microbiol Biotechnol*. 2005; 9:101–9. [PubMed: 16319499]
22. Kurupati P, Tan KS, Kumarasinghe G, Poh CL. Inhibition of gene expression and growth by antisense peptide nucleic acids in a multiresistant β -lactamase-producing *Klebsiella pneumoniae* strain. *Antimicrob Agents Chemother*. 2007; 51:805–11. [PubMed: 17158940]
23. Good L, Awasthi SK, Dryselius R, Larsson O, Nielsen PE. Bactericidal antisense effects of peptide–PNA conjugates. *Nat Biotechnol*. 2001; 19:360–4. [PubMed: 11283595]

Table 1

Cell-penetrating peptide–peptide nucleic acid (CPP-PNA) primary structure, description and gene target

Target gene	Description	CPP-PNA sequence ^a	PNA bases
<i>amp::lacZ</i>	<i>amp</i> promoter fused to <i>lacZ</i>	H-KFFKFFKFFK-O- cat actcttct-NH ₂	12
<i>rpoB</i>	Encodes the β -subunit of RNA polymerase	H-KFFKFFKFFK-O- cat cgctgctc-NH ₂	11
<i>asd</i>	Encodes aspartate-semialdehyde dehydrogenase needed for synthesis of diaminopimelic acid used to cross-link peptidoglycan	H-KFFKFFKFFK-O- cat ttttcgtct-NH ₂	12
<i>gyrA</i>	Encodes DNA gyrase, one of eight enzymes used in bacterial DNA replication	H-KFFKFFKFFK-O- cat atcgctcgga-NH ₂	12
<i>acpP</i>	Encodes acyl carrier protein (ACP) essential for fatty acid biosynthesis	H-KFFKFFKFFK-O-ct cat gctgg-NH ₂	10
<i>dnaG</i>	Encodes a protein primase that initiates DNA replication	H-KFFKFFKFFK-O- cat tacagatt-NH ₂	11
<i>polA</i>	Encodes DNA polymerase I necessary for DNA replication	H-KFFKFFKFFK-O-tt cat gcctgt-NH ₂	11
<i>kdtA</i>	3-Deoxy-D-manno-octulosonic-acid transferase; catalyses the transfer of 2-keto-3-deoxy-D-manno-octulosonic acid to lipid A	H-KFFKFFKFFK-O-gct cat ttcgct-NH ₂	11
<i>tsf</i>	Elongation factor Ts (EF-Ts) functions during the elongation stage of protein synthesis	H-KFFKFFKFFK-O- cat gtgtcgct-NH ₂	11

^aThe PNAs and CPP were synthesised (Panagene Inc., Daejeon, South Korea) and joined by an ethylene glycol linker designated as 'O'; a glycol linker of nine atoms used to distance the hybridisation portion of the molecule from the CPP. The bolded '**cat**' indicates the ATG start codon contained in the PNA sequence complementary to the specified gene.

Table 2Susceptibility of *Brucella suis* to peptide nucleic acids (PNAs) after 24 h of culture in tryptic soy broth

PNA (30 μ M)	Gene function	Log CFU/100 μ L/well ^a	Log reduction
No PNA, water ^b	Negative control	3.06 $\pm$ 0.12	0
Anti- <i>lacZ</i>	PNA-negative control	2.83 $\pm$ 0.13	0.23
<i>dnaG</i>	DNA replication	2.91 $\pm$ 0.02	0.15
<i>polA</i>	DNA replication	0.54 $\pm$ 0.09 *	2.52 *
<i>acpP</i>	Fatty acid synthesis	3.05 $\pm$ 0.10	0.01
<i>kdtA</i>	Fatty acid synthesis	1.56 $\pm$ 1.10 *	1.50 *
<i>tsf</i>	Protein synthesis	1.02 $\pm$ 0.92 *	2.04 *
No PNA, water ^c	Negative control	4.15 $\pm$ 0.07	0
<i>asd</i>	Cell wall	3.27 $\pm$ 0.09	0.80
<i>gyrA</i>	DNA replication	3.92 $\pm$ 0.06	0.23
<i>rpoB</i>	RNA synthesis	0 *	4.15

^aMean $\pm$ standard error in at least two separate experiments.^bThe first set of PNAs were tested at 10⁴ CFU/mL.^cIn a separate experiment, susceptibility to PNAs for *asd*, *gyrA* and *rpoB* was performed starting at 10⁵ CFU/mL.* Values found to be significantly different ($P = 0.05$) from the control by analysis using a two-tailed Student's *t*-test.

Table 3Effect of cell-penetrating peptide (CPP) versus peptide nucleic acid (PNA) linked to CPP on *Brucella suis*

Treatment	Log CFU/mL ^a	Log reduction
<i>B. suis</i> , no treatment	10.40 ± 0.11	0
20 µM CPP	9.93 ± 0.05	0.47
20 µM <i>polA</i>	0	10.40 *
25 µM <i>rpoB</i>	0	10.40 *

Cultures of *B. suis* were established in tryptic soy broth at 1.8×10^5 CFU/mL and were treated with different PNAs for 74 h at 37 °C.

^aValues represent the mean ± standard error.

* $P < 0.05$ using paired *t*-test.

Table 4Growth inhibitory concentration of *polA* peptide nucleic acid (PNA) in *Brucella suis*

<i>polA</i> (μM)	Log CFU/mL ^a	Log reduction
0	9.1 ± 0.21	0.0
10	9 ± 0.15	0.1
12.5	0	9.1 *
15	0	9.1 *
17.5	0	9.1 *
20	0	9.1 *

Cultures of *B. suis* were established in tryptic soy broth at 1.8×10^5 CFU/mL and were treated with different concentrations of *polA* PNA for 72 h. The longer duration of incubation allowed for validation of sustained growth inhibitory effect.

^aValues represent the mean ± standard error.

* Statistically significant at $P = 0.05$.

Table 5Efficacy of peptide nucleic acid (PNA) treatment in J774.A1 cells infected with *Brucella suis*^a

Treatment	Log CFU	Log reduction
Untreated	3.28 ± 0.18	0.00
Control (30 µM <i>lacZ</i>)	3.45 ± 0.31	+0.17
<i>asd</i> (30 µM)	1.84 ± 0.50	1.44 *
<i>gyrA</i> (30 µM)	2.47 ± 0.51	0.81 *
<i>acpP</i> (30 µM)	3.37 ± 0.18	+0.09
<i>dnaG</i> (30 µM)	1.78 ± 0.56	1.50 *
<i>polA</i> (30 µM)	2.27 ± 0.69	1.01 *

^a At 24 h post infection, the J774.A1 cell line was treated with various PNAs. After 24 h of treatment, cells were thoroughly washed, lysed and CFU of *B. suis* were determined (see Section 2.3). Each result represents the mean ± standard deviation of two independent experiments of infected macrophages cultured in duplicate.

* Values found to be significantly different ($P < 0.05$) from the control by analysis using a two-tailed Student's *t*-test.