

1 **Supplemental Information**

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3 **Supplemental Data:**

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5 **Table S1, related to Figure 1. Characterization of the most potent D-enantiomeric peptides**
6 **against *Pseudomonas aeruginosa* PA14 biofilms.** MBIC₅₀ values were determined using the 96-
7 well plate biofilm assay and correspond to the ability of the peptides to prevent biofilm formation
8 by 50%. The lowest MBIC₅₀ values obtained are shown in bold.

| Peptide name | Amino acid sequence | MIC (µg/ml) | MBIC₅₀ (µg/ml) |
|---------------------|----------------------------|--------------------|----------------------------------|
| DJK1 | VFLRRIRVIVIR | 20 | 10 |
| DJK2 | VFWRRIRVWVIR | 10 | 5 |
| DJK5 | VQWRAIRVRVIR | 16 | 1 |
| DJK6 | VQWRRIRVWVIR | 16 | 0.5 |
| RI-1002 | KRIRWVILWRQV | 10 | 5 |
| RI-1018 | RRWIRVAVILRV | 20 | 10 |

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11 **Table S2, related to Figure 1. MICs (for planktonic cells) and MBIC₅₀ (vs. biofilms; blue**
 12 **highlight) of various strains.** The strains included a high proportion of multi-drug resistant
 13 (MDR; resistant to >4 classes of antibiotics), XDR (resistant to most classes of antibiotics); DR
 14 resistant to 1-3 classes; and polymyxin resistant (PXR) isolates. NB. 1. The microtiter plate biofilm
 15 method underestimates activity by up to 10-fold relative to the flow cell method. 2. MBIC₅₀ was
 16 determined as the concentration reducing biofilm by 50% as there was not a clean cut-off as
 17 observed with planktonic cells but rather a steady decrease in biofilm formation over a 2-8 fold
 18 concentration range.

| Species | Bacterial strains | Phenotype | 1018 | | DJK5 | | DJK6 | |
|---------------------------------|-------------------|---------------------------------|-------------|--------------|-------------|--------------|-------------|--------------|
| | | | MIC (µg/ml) | MBIC (µg/ml) | MIC (µg/ml) | MBIC (µg/ml) | MIC (µg/ml) | MBIC (µg/ml) |
| <i>Acinetobacter baumannii</i> | ATTC 17987 | Wild type | >128 | 8 | 8 | 1 | 4 | 1 |
| | SENTRY C2 | XDR; PXR | >128 | 32 | 16 | 8 | 8 | 4 |
| | SENTRY C5 | MDR; PXR | >128 | 128 | 16 | 8 | 16 | 4 |
| | SENTRY C8 | DR; PXR | >128 | 2 | 8 | 4 | 8 | 2 |
| | SENTRY C11 | MDR; PXR | >128 | 64 | 8 | 4 | 8 | 4 |
| | SENTRY C12 | MDR; PXR | >128 | 128 | 16 | 8 | 8 | 4 |
| | SENTRY C13 | MDR; PXR | >128 | 64 | 16 | 8 | 16 | 4 |
| | SENTRY C14 | DR; PXR | >128 | 16 | 16 | 8 | 8 | 2 |
| | SENTRY C15 | MDR; PXR | >128 | 64 | 16 | 8 | 16 | 4 |
| SENTRY C87 | XDR; PXR | >128 | 4 | 8 | 4 | 4 | 1 | |
| <i>Burkholderia cenocepacia</i> | 4813 | MDR | >256 | 2 | >256 | 0.4 | >64 | 2 |
| <i>Enterobacter cloacae</i> | 218R | Derepressed Class C β-lactamase | 64 | 2 | 32 | 2 | 16 | 2 |
| <i>Escherichia coli</i> | 0157 | WT | 32 | 8 | 1.6 | 0.8 | 16 | 8 |
| | HB101 | WT | 32 | 16 | 16 | 4 | 8 | 2 |
| | VGH10 | MDR | 256 | 1 | 8 | 4 | 8 | 2 |
| | VGH11 | MDR | 64 | 32 | 16 | 4 | 8 | 8 |
| | VGH1 | MDR | 64 | 32 | 8 | 2 | 16 | 8 |
| <i>Klebsiella pneumoniae</i> | KPLN649 | WT | 32 | 1 | 3.2 | 1.6 | 4 | 2 |
| | 1549216 | WT | >64 | 8 | >64 | 2 | 32 | 2 |
| <i>Pseudomonas aeruginosa</i> | PA01 | WT | 64 | 8 | 16 | 2 | 16 | 1 |
| | PA14 | WT | 32 | 8 | 16 | 1 | 16 | 0.5 |
| | Brazil #21 | MDR; PXR | 64 | 8 | 16 | 4 | >16 | 16 |
| | Brazil #36 | MDR; PXR | 64 | 4 | 8 | 0.5 | 16 | 0.5 |
| | Brazil #172 | MDR; PXR | 64 | 32 | >16 | 0.5 | 16 | 1 |
| | Brazil #205 | MDR; PXR | 64 | 4 | 8 | 0.5 | 16 | 8 |
| | Brazil #211 | MDR; PXR | 64 | 32 | >16 | 0.5 | 8 | 0.5 |
| | LES400 - 27 | MDR | 16 | 2 | 8 | 2 | 4 | 1 |
| | LES400 - 30 | MDR | 16 | 1 | 8 | 1 | 4 | 0.5 |
| LES400 - 31 | MDR | 16 | 2 | 4 | 1 | 4 | 2 | |
| <i>Salmonella enterica</i> | C587 | WT | 64 | 3.2 | 3.2 | 0.8 | 4 | 1 |

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20 **Figure S1, related to Figure 4. Overproduction of (p)ppGpp led to decreased biofilm**
21 **susceptibility to D-enantiomeric peptides.** Addition of SHX, which leads to overproduction of
22 (p)ppGpp, resulted in the resistance of flow cell biofilm formation to 2.5 $\mu\text{g/ml}$ of peptide DJK-5.
23 After 3 days, bacteria were stained green with the all bacteria stain Syto-9 prior to confocal
24 imaging. Each panel shows reconstructions from the top in the large panel and sides in the right
25 and bottom panels (xy, yz and xz dimensions).

