Aggregating sequences that occur in many proteins constitute weak spots of bacterial proteostasis

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Aggregation is a sequence-specific process, nucleated by short aggregation-prone regions (APRs) that can be exploited to induce aggregation of proteins containing the same APR. Here, we find that most APRs are unique within a proteome, but that a small minority of APRs occur in many proteins. When aggregation is nucleated in bacteria by such frequently occurring APRs, it leads to massive and lethal inclusion body formation containing a large number of proteins. Buildup of bacterial resistance against these peptides is slow. In addition, the approach is effective against drug-resistant clinical isolates of *Escherichia coli* and *Acinetobacter baumannii*, reducing bacterial load in a murine bladder infection model. Our results indicate that redundant APRs are weak points of bacterial protein homeostasis and that targeting these may be an attractive antibacterial strategy.

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Loss of protein homeostasis\(^1\) is a constant threat for any living cell due to the highly crowded intracellular environment that brings into close proximity a large variety of polypeptides that need to undergo error-prone folding reactions in order to attain their native conformation\(^2\). To control this threat, cells have evolved a complex network of molecular chaperones, proteases, and other specialized molecules\(^3\). In spite of these cellular-response mechanisms, human protein-folding pathologies have made it clear that under persistent exposure to aggregating proteins, for example, as a result of mutation, protein homeostasis can eventually break down, which ultimately results in cell death\(^1\).

On the other hand, protein aggregation turns out to be a highly ordered and specific process: aggregation is more efficient between similar than between unrelated polypeptides\(^4\). At a mechanistic level, protein aggregation is mediated by short (between 5 and 15 residues) aggregation-prone sequence segments (called APRs), which on average occur at least once every 100 amino acids in the primary polypeptide sequence\(^5\). These APRs are generally sequence segments constituting the hydrophobic core of globular proteins or protein–protein interaction interfaces\(^6\). While forming the most stable part of the native protein, in unfolded proteins, APRs can also self-assemble with identical APRs from another protein to form \(\beta\)-structured aggregates\(^7\). The risk of aggregation is thus the highest during translation before the protein attains its native conformation\(^8\).

As the sequence of most APRs is unique within a given proteome\(^7\), aggregation will generally be restricted to identical proteins. However, a minority of APRs (or close homologs thereof) are found in several and sometimes many different proteins\(^9\). Given the sequence specificity of aggregation, this suggests that these proteins could coaggregate via such a common APR. The redundancy of these APRs therefore also suggests that they might constitute particularly vulnerable proteomic segments, and that under conditions of stress, these might act as hot spots for the initiation of proteostatic collapse.

In order to test this concept, here, we screen 125 aggregating sequences that have a high degree of redundancy in the \textit{Escherichia coli} proteome. In this manner, one peptide containing this APR could potentially affect the folding of many proteins containing highly similar APRs. Using this strategy, we identify several peptides that efficiently induce bactericidal protein aggregation and inclusion body (IB) formation in \textit{E. coli}. This process is bactericidal to \textit{E. coli} as well as \textit{Acinetobacter baumannii}, including clinical strains that are resistant to current antibiotics. Analyzing these peptides in more detail using several molecular and proteomic approaches, we find that these peptides induce widespread aggregation of bacterial proteins, resulting in bactericidal aggregation cascades involving hundreds of proteins. Moreover, the peptides effectively reduce bacterial load in a murine bladder \textit{E. coli} infection model, suggesting that redundant APRs in bacterial proteomes can be targeted for therapeutic purposes.

Results

Redundant aggregating sequences are rare. We used the statistical thermodynamics algorithm TANGO to analyze the aggregation propensity and APR redundancy of the \textit{E. coli} strain O157:H7 proteome. This yielded 3,535 APR sequences of at least six amino acids in length with a TANGO score of at least 20%. Given the length limitation of roughly 20 amino acids in solid-phase peptide synthesis with regard to yield and purity, our peptide design imposes a maximum length on the APRs that can be accommodated (APRs will be incorporated in a tandem repeat design peptide comprising twice the APR flanked by three gatekeeper arginine residues and linked by a single proline; see below), forcing us to restrict our experimental analysis to the 1,542 APRs with a length of seven amino acids. To analyze the redundancy of these sequences, we calculated for each of these APRs the number of times their sequence occurred in other proteins in the \textit{E. coli} proteome, considering zero, one, or two amino acid mutations (Fig. 1a). This shows that for more than 80% of the seven-residue APRs, their exact sequence is unique in the \textit{E. coli} proteome, while virtually no heptameric APR is found in more than five different proteins (Fig. 1a, red line). This observation might be related to the previous finding that selective pressure shapes sequence divergence in repeat-domain proteins such as titin in order to avoid interdomain aggregation\(^12\).

Allowing one mutation in the APR (85% APR sequence identity), we found that the number of unique APRs drops to 20% (Fig. 1a, blue line), but there is almost no heptameric APR sequence that possesses more than 10 APR homologs with a single-point mutation in other \textit{E. coli} proteins. This suggests that while one mutation is in many instances probably sufficient to avoid coaggregation (especially hydrophobic to charged-residue mutations), several single-point mutations will still allow for coaggregation (especially conservative hydrophobic mutations), which is confirmed by previous observations\(^9,13\). Finally, allowing for two mutations (70% APR sequence identity), we found that most APRs have more than 10 homologous APRs (Fig. 1a, green line); this suggests that at 70% sequence divergence (i.e., two mismatches in a heptameric sequence), coaggregation may be a rare occurrence, although it should still not be excluded. Indeed, Fig. 1a (green line) also suggests that high redundancy of two mismatch mutations (above 30) is still to be avoided. Very similar distributions of the number of homologous or identical APRs could also be observed in other bacterial proteomes, including \textit{Klebsiella pneumoniae}, \textit{Pseudomonas aeruginosa}, and \textit{A. baumannii} (Supplementary Fig. 1), suggesting that this is a universal feature of bacterial proteomes.

To experimentally investigate the impact of APR redundancy on the proteostatic robustness of the \textit{E. coli} proteome, we ranked APRs at the extreme of the redundancy distribution in Fig. 1a, showing up to 10 matches with one mismatch mutation and up to 100 matches with two mutations, and selected the first 75 most frequently occurring sequences from this list (Supplementary Table 1). The extreme values of these APRs are apparent from Fig. 1b, showing an enrichment of tail values of the \textit{E. coli} APR redundancy distribution displayed in Fig. 1a. Counting all amino acid substitutions as equal in terms of their likely \(\beta\)-interaction with the bait sequence is only a very rough approximation, but one that needs to be made since reliably separating amino acid substitutions that are conducive of \(\beta\)-interaction from those that are not is beyond the scope of current prediction algorithms.

Redundant APRs cause bacterial cell death. In order to generate efficient aggregation seeds, here, we employed a tandem repeat design previously validated\(^14,15\), in which the redundant APR is incorporated as a tandem repeat separated by a linker constituted by a single proline. In order to increase the colloidal stability of these aggregating peptides, the APRs are flanked by aggregation gatekeepers, a class of residues that was previously shown to reduce aggregation kinetics\(^8,16,17\). Since positively charged residues have also been shown to help bacterial uptake\(^18\), we selected arginine to obtain the following peptide layout: R-APR-RR (Fig. 1c). To further modulate the kinetics of aggregation of these peptides, we also added two variants of each of the first 25 peptides in the list by randomly mutating one residue in the first APR repeat to arginine (Supplementary Table 1). These 100 peptides were generated using solid-phase synthesis at 200-
nmol scale and dissolved in dimethyl sulfoxide (DMSO) to a theoretical stock concentration of 2 mM (by assuming 100% synthesis efficiency).

As we were looking for peptides capable of inducing a lethal proteostatic collapse, our primary screen consisted of measuring the effect of our peptides on the growth of *E. coli* O157:H7 at

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**Fig. 1** Proteome analysis, design, and screening of redundant APRs. **a** Distribution of the redundancy of APR sequences of length seven in the *E. coli* proteome: percentage of identical sequences (red), one mismatch (blue), and two mismatches (green). **b** Same distribution as in **a** for the 75 most redundant APRs in *E. coli*. **c** Design pattern for aggregating peptide screen. Tandem APRs are linked by a linker (a single proline residue) and embedded between gatekeeper residues (GK; arginine residues). **d**, **e** APR redundancy for toxic versus nontoxic peptides considering one (**d**) or two (**e**) mismatches. The bottom and top of the boxes are the first and third quartiles, and the band inside the box represents the median. The whiskers encompass the minimum and maximum of the data. Significant differences were computed using Welch’s *t* test. **f** Time-killing curve of selected peptides (P14, P2, and P5R) and ampicillin (Amp) against *E. coli* strain O157:H7 treated at MIC concentration (average and SD of three replicates). **g**–**i** Transmission electron microscopy (TEM) of cross-sections of resin-embedded *E. coli* O157:H7, treated for 2 h with buffer (**g**), P2 peptide (**h**), and P105 peptide (**i**) at MIC concentration. **j** Wide-field structured illumination microscopy (SIM) image of *E. coli* O157:H7 treated with P2 and stained with the amyloid-specific dye pFTAA (0.5 µM). **k** Monitoring of spontaneous buildup of resistance by monitoring the MIC value of *E. coli* O157:H7 cultures that are maintained on sublethal doses (50% of MIC) of selected peptides (P14, P2, and P105) or ampicillin (Amp) for 36 days.
dilutions of the peptide corresponding to concentrations of 1, 6, 12, and 25 μg/mL. Although no peptide inhibited bacterial growth at the highest dilution, 43 of them inhibited bacterial growth of E. coli O157 at 25 μg/mL, of which 11 were still active at 12 μg/mL and six had an apparent minimum inhibitory concentration (MIC) value of 6 μg/mL (Supplementary Table 2). We separated the original 75 APRs into two groups based on their inhibitor activity: the hits listed in Supplementary Table 2 and the inactive peptides. We then compared the sequence redundancy in the E. coli proteome of the APRs in both groups and found that the number of sequence matches found at both one and two mutations distance were significantly higher in the active group than in the inactive group (Fig. 1d, e). This shows that a high APR redundancy is associated to bacterial cell death, suggesting that these sequences could indeed represent proteomic weak spots of susceptibility for proteostatic collapse.

Bactericidal activity is associated to IB formation. In order to investigate whether APR redundancy does result in proteostatic collapse, we selected resynthesized and high-performance liquid chromatography (HPLC)-purified four positive peptides from the screen, P2, P5, P14, and P105, as well as three negative peptides, P3, P4, and P11, and confirmed their MIC and minimum bactericidal concentration (MBC) values (Table 1). Analysis of the rate of peptide bactericidal activity against E. coli O157:H7 (at MIC concentration) showed that the peptides exerted full bactericidal effect within 30 min to 2 h (Fig. 1f). Biophysical analysis of the P2 peptide in vitro, using mass spectrometry (MS), dynamic light scattering (DLS), Fourier transform infrared spectroscopy, transmission electron microscopy (TEM), and tinctorial assays confirmed the intrinsic aggregation propensity of the P2 peptide (Supplementary Note 1 and Supplementary Fig. 2). Cross-section TEM of peptide-treated bacteria revealed the widespread presence of large IBs, a hallmark of protein aggregation in E. coli, suggesting that the peptides act by interfering with bacterial proteostasis (Fig. 1g–i). These IBs, which are also called large polar aggregates, could be stained with pentameric formyl thiophene acetic acid (pFTAAs, Fig. 1j) and an extensively characterized amyloid-specific dye, which specifically binds to amyloid-like aggregates as well as disease-associated protein IBs. This IB-staining pattern could be observed for all bactericidal peptides, but not for any of the other peptides, although some other pFTAAs-positive structures could be discerned (Supplementary Fig. 3). We confirmed the cross-β-structure content of IB using thiolavin-T staining and correlative atomic force microscopy nanoimaging and Fourier transform infrared spectroscopy (Supplementary Note 2 and Supplementary Fig. 4). These data show that aggregation, resulting in cross-β-structure-enriched IBs, is a crucial property of the bactericidal peptide treatment.

When bacteria were repeatedly passaged on sublethal concentrations (50% of MIC) of the active peptides for a period of 36 days, no development of resistance was observed, whereas this was the case for the control antibiotic ampicillin (Fig. 1k). These data are supportive of a mode of action involving many targets throughout the E. coli proteome. To investigate this in more detail, peptide P2 was selected for further analysis. As a control, we generated a variant of P2, called P2Pro, in which we introduced proline substitutions at two positions in the APRs (Table 1), which conserve the hydrophobicity but disrupt the β-sheet propensity and hence reduce the aggregation propensity of the peptides. When we treated bacteria with the control peptides, we obtained MIC values of more than 200 μg/mL, indicating again that β-aggregation is key for the bactericidal effect of P2.

We derivatized P2 with fluorescein isothiocyanate (FITC) and established that the conjugate retained its antibacterial activity (MIC = 3 μg/mL against E. coli O157:H7) and quantified P2 uptake over time by flow cytometry (Fig. 2a–f). Analysis of P2 uptake by E. coli O157:H7 showed that after 15 min, 97.7 ± 2.9% (N = 4, mean and s.d.) of the cells are positive for FITC (Fig. 2b, f), increasing to nearly 100% after 1 h and beyond (Fig. 2c–f). In parallel, fluorescence microscopy of treated E. coli O157:H7 at MIC concentration confirmed no enrichment at the cell membrane of FITC-P2, but rather showed a clear accumulation of fluorescence in intracellular polar IBs from 15 min onward (Fig. 2g) that persisted at later time points (Fig. 2h) and thus confirmed the cross-section TEM images (Fig. 1g–i). Kinetics of bacterial cell death as measured by colony-forming unit (CFU) determination after P2 treatment (Fig. 2i) closely follow peptide internalization and coincide with the appearance of IBs after 15 min of treatment (50% after 15 min). On the other hand, bacterial cell death as monitored by propidium iodide (PI) uptake as a result of membrane permeabilization increased more slowly (2.1 ± 1.3% after 15 min to 85 ± 13.2% after 3 h, Fig. 2a–c, summarized in Fig. 2j, N = 4). This was confirmed by morphological analysis using scanning electron microscopy (SEM) (Supplementary Note 3 and Supplementary Fig. 5).

Together, these data demonstrate that P2 uptake and IB formation occur in close succession, and this is followed by cell death. Cell death precedes membrane disruption as significant growth inhibition is established coincidently with IB formation but before membrane permeability or deformation can be observed. Importantly, all PI-positive cells stain positive for aggregation by pFTAAs (Fig. 2k) and when bacteria were treated with FITC-P2Pro, which showed comparable uptake to P2 (Fig. 2l), no protein aggregation ensued (Fig. 2l) and hence no cell death could be detected (Fig. 2l). This again shows that aggregation of the peptide is essential to mediate its bactericidal effect.

### Table 1 MIC and MBC values of selected peptides purified by HPLC grade on E. coli O157

| Purified peptide | Sequences                      | MIC (μg/mL) | MBC (μg/mL) |
|-----------------|--------------------------------|-------------|-------------|
| P2              | RGLGLALVRRPRRGLGLALVRR         | 6           | 6           |
| P2Pro           | RGLGLAPPRRRGLGLAPRR            | >100        | >100        |
| P5              | RALLTTLRARPLLTTLRR             | 6           | 6           |
| PSR             | RRALLTLRIRPARRLLTRIR          | 12          | 12          |
| P105            | RALLTLRIRPARRLLTLRR            | 12          | 12          |
| P14             | RGLALLARRPRRGLALLARR           | 6           | 6           |

MIC minimum inhibitory concentration, MBC minimum bactericidal concentration, HPLC high-performance liquid chromatography.
effect on colony formation, showing that the stress in this condition is not lethal. In order to understand why P2-induced IB formation is irreversibly toxic, we compared the composition of IBs purified from *E. coli* O157:H7 cells treated with P2 at MIC concentration for 1 h with IBs purified from *E. coli* strain BL21 overnight overexpressing p53CD. Inspection of the resulting samples by TEM confirmed the successful purification of these IBs (Fig. 3c). The composition of IBs was subsequently analyzed by Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3d). The overall pattern of Coomassie staining revealed that a large number of similar bacterial proteins are trapped in the IBs of both P2-treated *E. coli*
Fig. 3 Inclusion body formation and proteostatic collapse. 

- **a** Growth curve of *E. coli* BL21-overexpressing p53CD (red) and control in the presence (green) or absence (blue) of P2 (average and SD of three replicates). p53CD bacterial growth in the presence of 0.4 mM IPTG.
- **b** Colony formation by *E. coli* BL21 p53CD-overexpressing bacteria. The bottom and top of the box are the first and third quartiles, and the band inside the box represents the median. The whiskers are drawn using Tukey’s method and show the extreme values that fall within 1.5 times the interquartile range.
- **c** Transmission electron microscopy image of an inclusion body from P2-treated *E. coli* O157:H7 (uranyl acetate).
- **d** Representative Coomassie blue SDS-PAGE of inclusion bodies from *E. coli* BL21-overexpressing p53CD (lane 1), mock (lane 2), and *E. coli* O157:H7 treated with P2 (lane 4), P2Pro (lane 5), or DMSO (lane 6). Molecular-weight markers are shown in lanes 3 and 7.
- **e** Western blot for dnaK, groEL, tig, and dnaJ of the same samples than that in **d**.
- **f** Fluorescence microscopy image of *E. coli* cells stably expressing a fluorescent fusion of DnaK (mCer) treated with P2 at MIC concentration.
- **g** Growth inhibition of cells treated with P2 with/without erythromycin (Erm, 100 μg/mL, average and SD of three replicates).
- **h** Percent of colony-forming units of chaperone-overexpressing *E. coli* strains treated by P2 peptide at MIC concentration for 1h. Significant differences from the WT are calculated using ordinary one-way ANOVA and Dunnett’s multiple-comparison test. Statistical significance is indicated as follows: **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.
E. coli O157:H7 and p53CD-overexpressing E. coli BL21, but not in untreated bacteria, suggesting a common molecular machinery associated with IB formation. In the p53CD IBs, the band corresponding to the molecular weight of p53CD and western blot is clearly visible using the p53 mouse monoclonal antibody pAb240 that recognizes a linear epitope located in the p53CD construct25 (Supplementary Fig. 6). Among the proteins trapped in both types of IBs, a number of molecular chaperones that are known to occur in IBs26 could be detected, including the bacterial heat shock protein 70 (Hsp70) homolog DnaK, the Hsp60 chaperonin GroEL, the ribosome-associated chaperone trigger factor (TF), and the bacterial Hsp40 DnaJ (Fig. 3e). The polar localization of a fluorescently traceable DnaK-mCerulean3 fusion protein (the latter moiety comprising a blue fluorescent protein) in E. coli K-12 MG1655 cells exposed to P2 confirms the association of DnaK with IBs (Fig. 3f).

We then investigated the Coomassie-stained SDS-PAGEs of IBs isolated both from E. coli O157:H7 and BL21:DE3 upon treatment with the remaining active and inactive peptides, which showed a similar high-intensity pattern of IB-associated proteins for the bactericidal peptides, which was absent or much reduced in the inactive peptides (Supplementary Fig. 7). So, when peptides with redundant APRs successfully induce aggregation in the cell, the process is bactericidal, but when a similar degree of aggregation is caused by the overexpression of a protein that is alien to this strain, it is not significantly toxic. These observations show that any toxicity that is associated with IB formation depends on the proteins that are aggregating into the IBs: a heterologous protein that aggregates does not represent a loss of an essential cellular function, and is hence not likely to be toxic. On the other hand, the simultaneous aggregation of many of the bacterial cell’s own proteins would eventually be expected to accumulate such high and pleiotropic levels of loss of function that cellular viability is ultimately irreversibly impaired. As a confirmation, we generated a tandem peptide that follows our design pattern, but that instead of a bacterial fragment contains an APR from p53CD and indeed found this not to be toxic to E. coli (Supplementary Note 4, Supplementary Figs. 7 and 8). This shows that aggregating peptides of this design are not toxic per se, but that their bactericidal effect depends on the induction of the aggregation of cellular proteins.

**Table 2 MIC of P2 for chaperone deletion strains**

| Gene deletion | MIC (µg/mL) | Protein name | Description |
|---------------|------------|--------------|-------------|
| KEIO WT       | 12         |              |             |
| Δ clpP        | 12         | ClpP         | Proteolytic subunit of the Clp protease |
| Δ clpA        | 12         | ClpA         | Substrate-specifying adapter for the Clp protease |
| Δ clpS        | 12         | ClpS         | Specificity adapter for the Clp protease (binds to and modulates ClpA) |
| Δ clpX        | 12         | ClpX         | ATP-binding subunit of the Clp protease |
| Δ lon         | 12         | Lon          | ATP-dependent protease, required for suppression of aggregation |
| Δ sUL4        | 12         | SulA         | Suppressor of Lon |
| Δ clpB        | 12         | ClpB         | Disaggregator of the Hsp100 family |
| Δ dnaK        | 6          | DnaK         | Folding chaperone of the Hsp70 family |
| Δ dnaJ        | 12         | DnaJ         | Co-chaperone to DnaK of the Hsp40 family |
| Δ grpE        | n/a        | GrpE         | Nucleotide exchange factor for DnaK |
| Δ htpG        | 12         | GroEL        | Folding chaperone of the Hsp90 family |
| Δ groL        | 12         | GroES        | Folding chaperone of the Hsp60 family |
| Δ groS        | n/a        | Hsp60        | Co-chaperone of Hsp60, of the Hsp10 family |
| Δ hslO        | 12         | Hsp33        | Oxidative stress-induced holdase |
| Δ hchA        | 6          | Hsp31        | Heat-dependent and temperature-stress-dependent holdase |
| Δ ibpA        | 12         | IbpA         | Small Hsp of the α-crystallin family |
| Δ ibpB        | 12         | IbpB         | Small Hsp of the α-crystallin family |
| Δ fig         | 12         | Trigger factor | Cotranslational folding chaperone, ribosome-associated |

**MIC minimum inhibitory concentration, WT wild type, Hsp heat shock protein**

**Cotranslational loss of protein homeostasis.** To gain more insight into the specific composition of IBs associated with the bactericidal activity of P2, we performed MS proteomic analysis. To achieve the highest possible coverage, we combined shotgun analysis of the entire IB samples with samples obtained from sectioning SDS-PAGE gels of P2-induced IBs into five equal sections and analyzed the protein composition of each by MS. We analyzed six independent biological repeats and considered them as hit proteins that were detected with a confidence of 99% in at least two of the six samples (Supplementary Data 1).

This analysis showed that, in agreement with the western blot, a wide range of chaperones can also be detected in the MS data. This included the cotranslational TF, the chaperonin system GroEL/GroES, the Hsp70 system dnaK/dnaJ/grpE, the Hsp90 homolog htpG, and the small Hsp ibpA, as well as the proteases lon and clpX/clpP/clpA (Supplementary Data 1). In total, 541 proteins were detected in the P2-induced IBs, suggesting that the bactericidal impact of P2 treatment corresponds to an extensive proteome-wide aggregation of proteins, in line with our initial design that aimed at inducing the collapse of protein homeostasis by aggregation of multiple proteins. Apart from the chaperones, the IBs were strongly enriched in ribosomal proteins (NCBI DAVID27, 47 genes, enrichment score of 26.12, Benjamini P value <10−98), indicating that protein aggregation in response to P2 may occur cotranslationally. To verify this hypothesis, we measured the MBC value of P2 in the presence of the macrolide antibiotic erythromycin, which is a bacteriostatic drug that acts by blocking the polypeptide exit channel in the ribosome. We observed a marked desensitization of bacteria (E. coli O157) (MBC > 100 µg/mL) to P2 after pretreating the cells with 100 µg/mL erythromycin for 2 h to block translation during peptide exposure, strongly supporting cotranslational induction of protein aggregation by P2 (Fig. 3g). In line with this, we observed by fluorescence-activated cell sorting (FACS) that there was no build up of pFTAA staining in the P2-treated bacteria in the presence of erythromycin (Supplementary Fig. 9), even though the uptake of FITC-P2 was not impaired by the presence of the ribosome inhibitor (Supplementary Fig. 10). These data show that in the absence of protein translation, there is no induction of protein aggregation and that this eliminates the bactericidal effect, again showing that the peptide needs to induce protein
aggregation in the bacteria to mediate cell death. The data in Supplementary Fig. 9 additionally demonstrate that the contribution of P2 itself to the pFTAAs staining is small compared to the bacterial proteins that aggregate in the IBs, meaning that the lack of pFTAAs staining in cells treated with P2Pro (Fig. 2i) really comes from the failure of this peptide to induce aggregation of bacterial proteins. In combination, the effect of erythromycin on P2 and the comparison of P2 with P2Pro establish a causal link between cotranslational induction of the aggregation of many bacterial proteins and the bactericidal effect of P2. Moreover, we could detect eight proteins that contain a sequence fragment similar to the APR of P2, including the HcaB protein from which the sequence was derived, and could integrate the other proteins in the IBs as nodes in an aggregation network connected by sequence-specific coaggregation edges (Supplementary Note 5, Supplementary Table 3, Supplementary Figs. 11, 12, 13, 14, and Supplementary Data 1).

If the bactericidal effect of P2 is indeed mediated by a loss of protein homeostasis, the cellular chaperone machinery would be expected to counteract or limit the effect of the peptide. In order to evaluate this, we determined the effect of P2 on 18 gene deletion strains for major bacterial chaperones and proteases in the E. coli K-12 BW25113 strain (taken from the KEIO collection29) and found that from the individual knockouts of the principal proteostatic components of E. coli, only the hsp70 dnaK and the small Hsp, hsp31, had a mild effect on the MIC value of P2 (Table 2). To ensure that this effect was not due to the reduced overall viability of the dnaK deletion strain, we also tested the MIC value at 30 °C and obtained the same result (MIC = 6 μg/mL). This confirms that the direct inhibition of chaperones and proteases is not the principal mechanism of action of P2. However, in several of the chaperone or protease deletion strains, the percent of bacterial cell survival after 1 h was significantly decreased for several deletion strains compared to the wild-type strain (Fig. 3h), showing that cell killing occurred much faster in the absence of certain chaperones. The deletion strains with the strongest effect largely matched those found in the IBs by MS, including those of the cotranslationally acting chaperones TF (tig), dnaK (Hsp70 family), and its cochaperone dnaJ, as well as the small Hsps hchA (Hsp31), hslO (Hsp33), and iphA (α-crystallin family). The disaggregase clpB and the aggregation-controlling protease lon also had strong effects. A similar picture emerged when we analyzed the sensitivity of E. coli BL21 after overexpression of selected chaperones: there was no effect on the MIC values, but the cell killing was slowed down compared to the wild-type strain in this experiment when the groEL/ES and dnaK/J/grpE systems were expressed in combination (Fig. 3i & Table 3). This demonstrates that the protein quality control (PQC) machinery temporarily opposes the aggregation induced by the P2 peptide, but is eventually overwhelmed and ends up associated to the aggregated proteins in the IB fraction.

**Table 3 MIC of P2 after chaperone overexpression in BL21**

| Plasmid | Overexpressed chaperone(s) | MIC (μg/mL) |
|---------|---------------------------|-------------|
| pG-KE8  | dnaK, dnaJ-grpE, groES-groEL | 25          |
| pGro7   | groES-groEL               | 25          |
| pKE7    | dnaK, dnaJ-grpE            | 25          |
| pTG12   | groES-groEL-tig            | 25          |
| pTf16   | tig                        | 25          |
| wt      | —                          | 25          |

MIC minimum inhibitory concentration

**IB composition versus toxicity.** To better understand the importance of the quantity and identity of proteins pulled into aggregates in distinguishing between lethal aggregation and controlled, nontoxic IB formation, we performed a large-scale MS proteomics experiment, comparing IB composition from control conditions to those formed upon overexpression of p53CD with treatment with toxic (P2, P5, and P14) and nontoxic peptides (P2Pro, P4), as detailed in Supplementary Note 6, Supplementary Data 2 and 3, and Supplementary Fig. 15. The first observation that stands out in this analysis is that IBs associated with lethal conditions contain significantly more proteins than those associated to nontoxic conditions (Supplementary Fig. 16A). A common core of IBs was defined by identifying a large group of 424 proteins that are present in IBs of untreated, as well as toxic and nontoxic peptide-treated BL21. This common core appears to be primarily composed of the molecular machinery required to mediate and control IB formation, as well as other proteins that appear to associate to IBs for reasons that are less obvious (Supplementary Fig. 17A and B). The former category comprises the molecular chaperones, such as the chaperonin groE, the bacterial hsp70 dnaK, the disaggregase clpB, the cotranslational foldase TF, and, to a lesser extent, the bacterial Hsp90 htpG among others (Supplementary Fig. 18).

All structural constituents of the ribosome and other elements involved in the control of protein translation also commonly dominate the formation of IBs irrespective of the peptide treatment (Supplementary Fig. 17C and D). However, in addition to this common core of proteins, each IB contains an additional set of polypeptides specifically associated to each condition (Supplementary Fig. 14B). Of all the samples, the IBs resulting from the overexpression of the p53CD protein contain the smallest number of additional proteins (eight proteins, including p53CD). The p53CD polypeptide strongly dominates the composition of the IBs from the overexpressing cells (Fig. 3d).

This indicates that the heterologous expression of p53CD leads to IBs that essentially consist of a large quantity of the overexpressed protein, plus the proteins typically found in the IB fraction across conditions. This makes sense in the context of recombinant protein purification from such IBs and explains the lack of toxicity observed under these conditions. It is also in line with the notion that p53CD aggregation constitutes a proteostatic stress that, contrary to P2, does not cause a proteostatic collapse and is not lethal to the bacterial cell. In the IBs from nonlethal conditions, including p53CD, P2Pro, and P4, we detect fewer additional proteins than in the IBs from lethal conditions (Supplementary Fig. 16B). Moreover, these additional proteins in the nonlethal IBs are often from the same functional categories as the GO enrichments associated to the common core, suggesting that the formation of nontoxic IBs is a more controlled process than those formed in the lethal conditions (Supplementary Fig. 17). In the IBs from lethal conditions combined (P2, P5, and P14), we found between 47 and 154 bacterial proteins in addition to the typical IB proteins found in the common core, with contrasting molecular functions (Supplementary Fig. 17C), showing what a devastating impact these peptides have on the PQC, and confirming the notion that peptides containing redundant APRs cause widespread protein aggregation. The isolated genetic deletion of many of these elements individually completely impairs viability. So, it may be unsurprising that the accumulated loss of many of these proteins is lethal, even if the knockdown of each individual protein is likely to be less complete than in the genetic deletion.

In conclusion, the MS data presented here are in good agreement with the notion that our peptides cause a pleiotropic and accumulated loss of function to protein aggregation that is eventually lethal.
Exploiting redundant APRs in vivo. Since bioinformatics analysis showed that our peptides differ markedly from existing antimicrobial peptides (Supplementary Note 7), we wanted to know if the induction of proteostatic collapse mediated by P2 could be exploited as an antimicrobial peptide.

We tested the uptake of P2 in other bacterial strains and found peptide uptake in several different bacterial species (Supplementary Fig. 19). Then, we proceeded to test the activity of P2 against 17 clinical isolates of *E. coli* and 15 of *A. baumannii*, which displayed a varying spectrum of resistance against well-established antibiotics (Supplementary Table 4). We found P2 to be effective on 16 out of 17 of the tested *E. coli* strains and 14 out of 15 of the *Acinetobacter* strains, including those resistant to the last-resort carbapenems. As a first indication of specificity of the peptides, we demonstrated that there were no hemolytic-to-human erythrocytes (Fig. 4a), suggesting that the bactericidal effect of P2 is not the result of a generic toxicity. This was further confirmed by CellTiter Blue (Fig. 4b) and lactate dehydrogenase (LDH) release (Fig. 4c) assays to assess the cytotoxicity of the peptide for HeLa cells. The specificity of P2 for *E. coli* O157:H7 was estimated by determining the concentration at which bacterial growth is 50% inhibited (IC50 = 1.5 μg/mL) and compared to the concentration at which the peptide induces 50% lysis of human erythrocytes (LC50 = 1,100 μg/mL), yielding...
Fig. 4 Cross-seeding and in vivo activity. a Concentration-dependent hemolysis of human erythrocytes by selected peptides (average and SD of three replicates) shown as percent of hemolysis compared to 1% Triton. b, c Cytotoxicity of P2 (black bars) and P2Pro (gray) to human HeLa cells measured using the CellTiter Blue assay (b) (average and SD of three replicates) and the lactate dehydrogenase (LDH) release assay (average and SD of three replicates), represented as percentage of cell survival compared to control. d Fluorescence micrography of HeLa cells mixed with E. coli O157:H7, treated with FITC-P2 (green channel). Blue is DAPI (4',6-diamidino-2-phenylindole), red is CellMask Deep Red. e Aggregation kinetics of the Alzheimer β (Aβ) peptide at 50 μM with/without P2, monitored using thioflavin-T fluorescence (average and s.d. of three replicates). f Same as b for human islet amyloid polypeptide (IAPP). g Inhibitory effect of 5, 25, and 50 μg/mL P2 on bacterial growth in the presence of human blood serum (25% or 50%; average and SD of three replicates). h ELISA on immobilized FITC-P2 using blood serum of mice treated for 18 days with 30 mg/kg P2. An anti-FITC antibody was used as a positive control for peptide immobilization (three replicates from three mice). i–l Antibacterial efficacy of P2 in a mouse model of bladder infection. The bacterial load of mice infected with E. coli O157:H7 transurethrally was determined after treatment with P2 (P2 administered urethrally (P2 UT) or intraperitoneally (P2 IP)) and controls (ampicillin administered orally (Amp.oral), buffer (mock), and P2Pro administered urethrally (P2Pro2UT)) in i kidney, j colon, k bladder, and l ureter. Each treatment group consisted of 15 animals. Bacterial loads are expressed as log_{10}(CFU/mL). See text and Methods for more details. Plots i–l show individual measurements, as well as mean and s.d. Significant differences were calculated using ANOVA with Tukey’s post hoc test. Statistical significance is indicated as follows: ***P ≤ 0.001.

an apparent therapeutic ratio of 730. In order to test the in vivo potential of P2, we treated a coculture of mammalian (HeLa) cells and E. coli O157:H7 with P2 and observed the preferential accumulation of P2 in bacteria but not in mammalian cells (Fig. 4d). In addition, we tested the cross-reactivity of P2 aggregation with known disease-associated amyloidogenic peptides in vitro by spiking P2 into freshly dissolved preparations of the human Alzheimer β (Aβ) peptide or the human islet amyloid polypeptide and did not observe an increase of the rate of aggregation of these peptides (Fig. 4e, f), showing that the peptide is not a general inducer of protein aggregation. We did, however, observe a delay in the aggregation onset of the Aβ peptide, suggesting that a transient interaction did take place (Fig. 4e). Finally, we found that P2 incubated in 25 or 50% human serum for 2 h was still able to inhibit bacterial growth at 25 and 50 μg/mL (Fig. 4g). Moreover, initial experiments outlined in Supplementary Note 8 showed that mice tolerated P2 treatments well (Supplementary Tables 5, 6 and Supplementary Figs. 20, 21, and 22 and Fig. 4h).

Based on these observations, we tested the antibacterial efficacy of the P2 in a mouse bladder infection model. In this model, an inoculum of 50 CFU/mL suspension of E. coli O157:H7 was delivered via the urethra to the bladder of healthy Swiss mice. One hour post infection, we administered a single injection of P2 at 10 mg/kg, either via the urethra (denoted at UT, n = 15) or intraperitoneally (denoted as IP, n = 15). As a positive control, we included ampicillin treatment, which was administered orally (20 mg/kg), while P2Pro was administered urethrally (10 mg/kg) and buffer treatment served as negative controls. Twenty-four hours after treatment, the animals were killed and the bacterial titer in kidney, colon, bladder, and ureter was determined by plating the macerated tissue (Fig. 4i–l). These experiments revealed a significant reduction of the bacterial titer in the different organs of P2-treated animals (P value <10^{-4} compared to buffer control and P value <10^{-4} compared to nonaggregating P2Pro control, analysis of variance (ANOVA) with Tukey’s posttest). The log-fold reduction of the average bacterial load ranged from 2.3 in the ureter to 3.0 in the kidney after IP delivery and from 2.6 in the colon to 3.1 in the ureter upon UT delivery. The effect was comparable to orally dosed ampicillin (20 mg/kg) in the ureter, but P2 treatment was better in reduction of the bacterial load in the other tissues, ranging from 1.48 log-fold in the colon to 2.0 log-fold in the bladder. These results clearly indicate that the antimicrobial activity of P2 against E. coli is maintained in vivo.

Discussion

The emergence of multidrug-resistant Gram-negative infections represents one of the major healthcare challenges of the coming decade(s), but alternative treatment options are currently not available.29 We present here a class of peptides with a strong bactericidal effect against multidrug-resistant clinical isolates of E. coli and A. baumannii, both in vitro and in a bladder infection model in the mouse. These peptides act by inducing widespread protein aggregation in these bacteria, eventually causing cell death by overcoming the bacterial protein homeostasis system. Protein misfolding and aggregation are relatively common events under normal physiological conditions and are increased under conditions of stress such as heat shock, but the PQC controls the process and avoids that it degenerates. For example, upon recombinant production of heterologous proteins in bacteria, the aggregating protein is stored into IBs, and little or no toxicity is associated. However, human protein aggregation diseases revealed that when stress is too intense or sustained, the capacity of the PQC to control misfolding events is exceeded, resulting in protein aggregation and IB formation.30–32 Here, we exploit this concept to induce toxic protein aggregation in the Gram-negative bacteria by using aggregation-prone peptides whose sequence is based on aggregation protein sequences that occur in many bacterial proteins. The idea is that these peptides will cause aggregation of many different bacterial proteins that share this short seven-amino-acid stretch. The pleiotropic loss of function of many proteins at the same time eventually overcomes the capacity of the PQC to correct the problem and the viability of the cell is negatively affected. Because the approach disrupts an essential process by targeting many different proteins, we hope that the emergence of resistance may be inherently more difficult for the bacteria than for single-target approaches. Indeed, repeated passaging of bacteria on sublethal concentrations of the active peptides for a period of 36 days did not result in the development of resistance contrary to the control of the antibiotic ampicillin.

Our method is based on the notion that protein aggregation is a sequence-specific process that is nucleated by, and can thus be induced with, short APRs within a protein that self-assemble to form aggregates.8,33–35 Most proteins possess at least one APR in their sequence. We recently demonstrated, however, that most of these aggregation-prone sequences are unique in a proteome.7,10 In other words, when a protein aggregates, it will generally only aggregate with identical proteins. We previously exploited the fact that aggregation is sequence specific and that most aggregating sequences are sparse in a proteome to induce specific protein knockdown of target proteins in plants36 and mammalian cells37,38 or to achieve protein detection in western blot using protein-specific APRs.2 During these exercises, we realized, however, that a minority of aggregation-prone sequences are found within several and sometimes many proteins. By the same mechanistic reasoning, this suggests that a minority of proteins will, when
aggregating, induce the aggregation of several and even many other proteins. This also suggests that most proteomes possess proteostatic weaknesses that might constitute hot spots for proteostatic collapse under conditions of stress.

From a small screen of 75 frequently occurring APRs from *E. coli*, we found that more than half had antibacterial activity at 25 μg/mL, showing that these APRs are a particularly rich source of APRs that can induce widespread protein aggregation. For several of these peptides, we demonstrated that they indeed enter cells and cause protein aggregation in the form of IBs that contain hundreds of bacterial proteins. Taken together, our findings suggest that redundant APRs (which are a minority of the APRs in the *E. coli* proteome) indeed represent hot spots for proteostatic collapse, the aggregation of which is so widespread that it is bactericidal. This approach could therefore also represent an interesting paradigm to be explored for the development of a new class of antibiotics.

Methods

All primer sequences are listed in Supplementary Table 7.

Bioinformatics analysis. Protein sequences for various bacterial strains were obtained from UniProt® and redundant was removed using the cd-hit algorithm®. We employed the software algorithm TANGO to identify APRs across this work, using a TANGO score of 5 per residue as the lower threshold. This was previously shown to yield a Matthews correlation coefficient of 0.892 between experimentally determined and predicted aggregation. The parameter configuration TANGO was temperature at 298 K, pH at 7.5, and ionic strength at 0.1 M.

Peptide synthesis. Initial peptide screens were obtained as microscale peptide sets (200-nmol scale) from JPT (Berlin, Germany). Peptide hits were reordered from several vendors (Genscript, Shanghai, China and PepScan, Leestad, The Netherlands) at higher purity (>90%) and were also produced in-house using the Intavis Multipep R® automated synthesizer. In-house HPLC purification was performed with a Zorbax SB-C3 semi-preparative column (Agilent, USA) installed on a Prominent HPLC (Shimadzu, Japan). Peptides were freeze-dried and stored at −20°C prior to use.

Bacterial strains and growth conditions. Bacterial cells were collected from different human clinical samples, and from the University Hospital Leuven-Gasthuisberg. Gram-negative bacterial strains were cultivated in Luria-Bertani (LB) broth (Difco) and Gram-positive bacteria strains were grown in a rich medium, brain heart infusion broth (Difco, Sparks, MD, USA) at 37°C. Whenever required, growth media were supplemented with appropriate antibiotic to the medium or plates (ampicillin 25 μg/mL, erythromycin 100 μg/mL, chloramphenicol 20 μg/mL, kanamycin 30 μg/mL, t-arabinose 0.5 mg/mL, and tetracycline 2mg/mL). Escherichia coli DH5α (Thermo Fisher Scientific) was used for cloning and plasmid amplification. For selection of antibiotic resistance colonies, *E. coli* carrying plasmids was grown in LB medium supplemented with the relevant antibiotic. Bacterial CFU counting was done on blood agar plates (BD Biosciences) or LB agar plates. Species identification and antibiograms for all clinical isolates were performed using MALDI-TOF and VITEK® 2 automated system (bioMérieux).

MIC determination. The MICs of active peptides were determined via the broth microdilution assay according to the EUCAST guideline, which was performed in 96-well polystyrene flat bottom microtiter plates (BD Biosciences). Briefly, a single colony was inoculated into 5 mL LB medium and grown to the end-exponential growth phase in a shaking incubator at 37°C. Cultures were subsequently diluted to an OD₆₀₀ of 0.002 (1 × 10⁸ CFU/mL) in fresh LB medium. To evaluate the effect of aggregators over time, bacterial cells were subjected to a concentration of different peptides at the MIC value for different periods of time (5 min, 10 min, 30 min, 1 h, 6 h). After the defined contact period, 50 μL of each culture was serially diluted and plated on blood agar plates. Plates were incubated overnight at 37° C without shaking. The number of viable organisms was counted as CFU/mL.

Antibody and antibiotic product codes. The antibodies and antibiotic product codes used are as follows: anti-CLPB (Aviva, catalog# ARS3790_0900) 0.5 μg/mL, anti-Dnak (Aviva, catalog# OAE001) 1 μg/mL, anti-TF (Clontech, catalog# M201) 2 μg/mL, anti-gröEL (Abcam, catalog# ab2592) 1 μg/mL, and anti-Dnal (Enzo Life Sciences, catalog# AD1-SPA-410-D) 0.5 μg/mL, Ampicillin sodium, CAS number 69-52-3 (Duchefa Biochemie, catalog# A0104), erythromycin, CAS number 114-07-8 (Sigma-Aldrich, catalog# E5389), chloramphenicol, CAS number 56-78-7 (Duchefa Biochemie), and kanamycin CAS number 56-75-7 (Duchefa Biochemie).

Biophysical characterization. DLS measurements were performed at a ambient temperature using a DynaPro DLS plate reader (Wyatt, Santa Barbara, CA, USA), employing a 800 nm laser at 90° angle in flat-bottomed 96-well microclear plates (Greiner, Frickenhausen, Germany). Data were recorded in 10 s reads and 40 readings were averaged. All calculations of hydrodynamic radius were performed using the Wyatt Dynamics software. For attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR), we used the Bruker Tensor 27 infrared spectrophotometer and its Bio-ATR II accessory. We used a spectral resolution of 4 cm⁻¹ and recorded spectra in the 900–3,500 cm⁻¹ interval, averaging over 120 data acquisitions while purging the instrument with dry air. Atmospheric interference corrections and baseline subtractions were carried out before the spectra were rescaled in the amide II area (1,500–1,600 cm⁻¹). For TEM, samples were adsorbed on carbon-coated Formvar 400-mesh copper grids (Agar Scientific, England) for 5 min, washed, and stained with 1% (v/v) uranyl acetate. Electron micrographs were recorded using a JEOL JEM-1400 microscope (JEOL, Tokyo, Japan) at 80 kV.

Time-killing kinetic assay. The time-killing kinetic study of the peptides was carried out to assess the killing rate of the bacteria at enough exposure time points. This study was done according to standard guide for assessment of antimicrobial activity using time-killing kinetic procedure. Selection of agent concentrations was guided by MIC endpoints.

Briefly, 20 μL of frozen cultures of *E. coli* O157: H7 were inoculated into 5 mL LB and grown to the end-exponential growth phase in a shaking incubator at 37°C. Cultures were subsequently diluted to an OD₆₅₀ of 0.002 (1 × 10⁸ CFU/mL) in fresh LB medium. To evaluate the effect of aggregators over time, bacterial cells were subjected to a concentration of different peptides at the MIC value for different periods of time (5 min, 10 min, 30 min, 1 h, 6 h). After the defined contact period, 50 μL of each culture was serially diluted and plated on blood agar plates. Plates were incubated overnight at 37°C without shaking. The number of viable organisms was counted as CFU/mL.

Multistep resistance development study. The ability of the target strains to develop resistance to active compounds was evaluated by repeated subculturing in the presence of the half-MIC value of the active peptide over 30 days. Briefly, *E. coli* O157 cultures were grown in LB medium, the OD of bacteria was then adjusted to an OD₆₅₀ of 0.002 (equivalent to 1 × 10⁸ CFU/mL). Bacterial cells were treated by each aggregator at half MIC concentration; after a 24 h incubation period, the MICs were tested by a microdilution assay according to the EUCAST guideline and the bacteria were re-cultured in the presence of the half-MIC value of the respective aggregator. Ampicillin was used as the positive control in this experiment.

Scanning electron microscopy. For SEM, *E. coli* O157 or BL21 bacterial cells in end-exponential growth phase were diluted to a density of 10⁶ CFU/mL and treated with supra-MIC concentrations of peptides. After 2 h treatment, bacterial cells were trapped by nitrocellulose membrane filters (0.1 μm, CAS 900470.0 Ref. VCWPO/ 300) and then were fixed with 2% glutaraldehyde for 1 h. One percent of 1% osmium tetroxide (OsO₄) was used as postfixation in 0.1 M sodium cacodylate buffer for 1 h. Samples were washed three times with cacodylate buffer (0.1 M sodium cacodylate) for 10 min at room temperature (RT). The samples were dehydrated with a graded ethanol series (50, 70, 90, and 100% alcohol). After the dehyration step, samples were dried by hexamethyldisilazane for 1 h and mounted on the specimen stubs and sputter coated with gold. An SEM-FEG (field emission guns) microscope (JEOL JSM 6700F) with an accelerating voltage of 30 kV was used.

Cross-section TEM. Escherichia coli at the end-exponential growth phase were washed twice and diluted with physiological water and subsequently treated with either MIC value of specific aggregator peptides or buffer for 2 h (Control group) at 37°C. After 2 h, bacterial cells were centrifuged at 4,000 × g for 4 min and pellets were re-suspended in 0.15 M Na-cacodylate buffer, pH = 7.2–7.4 (+2.5 ± 0.1 M CaCl₂ + 1 mM MgCl₂), for 1 h. Then, the pellets were washed with cacodylate buffer, re-suspended in 1.5% low melting point agarose (Sigma A4018) in cacodylate buffer (40°C), and centrifuged at 4,000 × g for 4 min. The centrifuge tubes were placed on ice for 15 min, after which the tips containing the pellets were cut off. The pellets were re-suspended in a drop of cacodylate buffer. Pellets were cut into 1 mm³ cubes (4°C), post-fixed with 1% OsO₄ in distilled water for 2 h, and washed twice with distilled water. Thereupon, the samples were dehydrated in a
graded ethanol series (30, 50, 70, 90, and 100%) for 5 min in each step at 4 °C while slowly rotating (ethanol 100%, repeated three times). Finally, cells were treated with propylene oxide (Sigma CAS number: 75-56-9) twice for 15 min at 4 °C, infiltrated with a 1:1 mixture of epoxy resin and propylene oxide (60 min at 4 °C, slowly rotating), and subsequently left in a mixture of 2:1 epoxy resin and propylene oxide overnight under a fume hood without caps. The next morning, samples were placed in 100% fresh epoxy resin, embedded in BEEM capsules in the evening, and polymerized overnight at 60 °C. For light microscopy, sections were cut with a Leica ultratcut UCT ultramicrotome and observed in a JEOL JEM-1400 TEM operated at 80 kV and equipped with an Olympus Quemera 11Mpxl camera.

**In vitro hemolytic activity test.** The hemolytic activities of peptides were determined by hemolysis against human erythrocytes. Pooled fresh blood was obtained from healthy volunteers (Red Cross Flanders) and erythrocytes were collected by centrifugation 1,000 × g for 5 min (anticoagulated by EDTAK). The pellet was washed three times with phosphate-buffered saline (PBS) and was diluted to a concentration of 8% in PBS. One hundred microliters of 8% red blood cells solution was mixed with 100 μL of serial dilutions of peptides in PBS buffer in 96-well plates (BD Biosciences). The reaction mixtures were incubated for 1 h at 37 °C. Thereupon, the plate was centrifuged for 10 min at 1,000 × g and 100 μL of supernatant was transferred to a sterilized 96-well plate (BD Biosciences, bottom). The release of hemoglobin was determined by measuring the absorbance of the supernatant at 405 nm. The hemolytic activity was determined as the minimal peptide concentration that caused hemolysis (minimal hemolytic concentration). Erythrocytes in 1% Triton and maximum used concentration of vehicle were used as the control of 100% and 0% hemolysis, respectively.

**In vitro mammalian cytotoxicity.** Mammalian cytotoxicity was measured using the LDH release method (Roche, Mannheim, Germany) and CellTiter Blue (Promega) methods. Briefly, HeLa cells (the human cervix epithelial carcinoma cell line HeLa was obtained from the European Collection of Authenticated Cell Cultures (ECACC 93021013)) were seeded in 96-well round bottom plates at a concentration of 3 × 10^4 cells/mL in Dulbecco’s modified Eagle’s medium and treated by different concentrations of peptides. Cells treated with 1% Triton™ X-100 and vehicle were considered as positive and negative controls, respectively. Microplates were incubated at 37 °C with 5% CO_2 and 90% humidity for 4 h. The micro-plate was centrifuged at 200 × g for 10 min. One hundred microliters of supernatant was transferred to a clear 96-well flat-bottom microplates. In order to determine LDH activity in the supernatants, 100 μl reaction mix (catalyst and dye solution) was added to each well and incubated for 30 min at RT in the dark and the LDH activity in the supernatants, 100 μl reaction mix (catalyst and dye solution) was added to each well and incubated for 30 min at RT in the dark and the LDH activity was determined by measuring the absorbance of the samples was measured at 490 nm. The cell viability was calculated using the formula: 
\[ \text{cell viability} = \frac{\text{positive control value} - \text{negative control value}}{\text{positive control value}} \times 100. \]

The amount of absorbance is proportional to the number of living cells and corresponds to the cells’ metabolic activity. Cell lines were regularly tested for Mycoplasma contamination.

**Expression of HcaB in E. coli ATCC 25922 strain.** The coding regions of E. coli O157 HcaB were amplified using ncaB-specific primers (5'-ATTGTCGA-CATGAGCGATCTGCATAACGA-3' and 5'-ATGTGAGCGATTTATCGAAGAAGGC-3') and treated with restriction enzyme digestion, colony PCR, and nucleotide sequence analysis of the insert and then by sequencing.

**Knockout and co-expression chaperone strains.** Knockout chaperon strains were purchased from ThermoFisher Scientific (catalog# E. coli O157 strains). A single deletion mutant in each deletion was used. Each deleted gene has been replaced by a kanamycin (30 μg/mL) resistance cassette. Each deleted gene in each strain was confirmed by restriction enzyme digestion, colony PCR, and nucleotide sequence analysis of the insert and then by sequencing.

**Macrolide and peptide interaction.** To evaluate the effect of peptides in the presence of erythromycin, E. coli O157 was grown in 5 ml of LB. Exponential-phase cultures were then diluted to 10^8 cells/mL. Bacterial cells were treated with the erythromycin at the concentration of 100 μg/mL for 3 h to stop growing at 37 °C, without shaking. Different concentrations of peptides (from 100 to 0.75 μg/mL) or buffer were placed in 96-well plates with at least three replicate wells (30 μL). Fifty microliters of erythromycin-treated bacteria were added to each well and 96-well plate was incubated at 37 °C for 10 min. The bacterial pellets were serially diluted and cultured on blood agar plates. Plates were incubated at 37 °C overnight. The number of living cells was measured by CFU counting method. Flow cytometry analysis of bacteria treated by peptides. Using a double staining technique with propidium iodide (PI) and FITC peptides, killing rate and peptide uptake were evaluated in a two-dimensional analysis. Briefly, end- exponential growth phase E. coli O157 cells (10^6 CFU/mL) were washed and treated with peptides (P2 or Pro2/ FITC labeled) at MIC value for different times (30 min, 1 h, and overnight). Alexa647-labeled DAPI was added to the bacteria, and after incubation for 5 min, the mixture was aliquoted (500 μL) into FACS tubes. Flow cytometry analysis of bacteria treated by peptides. Using a double staining technique with propidium iodide (PI) and FITC peptides, killing rate and peptide uptake were evaluated in a two-dimensional analysis. Briefly, end-exponential growth phase E. coli O157 cells (10^6 CFU/mL) were washed and treated with peptides (P2 or Pro2/ FITC labeled) at MIC value for different times (30 min, 1 h, and overnight). Alexa647-labeled DAPI was added to the bacteria, and after incubation for 5 min, the mixture was aliquoted (500 μL) into FACS tubes. To correlate the activity of the peptides with cell death, the fluorescence intensity was measured in two channels using the GalliosTM Flow Cytometer. PI: excitation 536 nm and emission 677 nm, FITC: excitation 490 nm and emission 525 nm. Heated bacteria at 90 °C for 10 min were used as PI-positive control.

**IB purification.** Twenty milliliters of overnight culture of bacteria was centrifuged for 30 min at 4,000 × g and washed by physiological water. Bacterial cells were treated by peptide at MIC for half killing time; afterwards, the bacterial pellets were washed with 10 mL buffer A (50 mM HEPES, pH 7.5, 300 mM NaCl, 5 mM β-mercaptoethanol, 1.0 mM EDTA) and centrifuged at 4 °C for 30 min at 4,000 × g. The supernatant was discarded, and 20 mL of buffer B (buffer A plus 1 μg/mL leupeptin, 0.1 mg/mL aBEEFS (4-2-aminophylybenzenesulfonyl fluoride hydrochloride)) was added to the bacterial pellet. In order to break the cells, a Glen Creston Cell Homogenizer with pressure set to 20,000-25,000 psi was used, and in addition, the suspensions were sonicated (Branson Digital sonifier 50/60 HZ) on ice with alternating 2 min cycle (15 pulses at 50% power with 30 s pauses on ice, until completing 2 min total sonication time). The lysed cells were centrifuged at 4 °C for 30 min at 11,000 × g. The precipitated fraction was re-suspended with 10 mL buffer D (buffer A plus 0.8% (V/V) Triton X-100, 0.1% sodium deoxycholate) and the suspension was sonicated to ensure the pellet is completely dissolved. This step repeated three times. Centrifugation was performed at 4 °C for 30 min at 11,000 × g. Finally, to solubilize IB, the pellet was suspended in 1 mL of buffer F (50 mM HEPES, pH 7.5, 8.0 M urea) per gram of precipitated fraction.

**Peptide activity and stability in the presence of serum.** Bacterial cells were treated by peptide at MIC for half killing time; afterwards, the bacterial pellets were washed with 10 mL buffer A (50 mM HEPES, pH 7.5, 300 mM NaCl, 5 mM β-mercaptoethanol, 1.0 mM EDTA) and centrifuged at 4 °C for 30 min at 4,000 × g. The supernatant was discarded, and 20 mL of buffer B (buffer A plus 1 μg/mL leupeptin, 0.1 mg/mL aBEEFS (4-2-aminophylybenzenesulfonyl fluoride hydrochloride)) was added to the bacterial pellet. In order to break the cells, a Glen Creston Cell Homogenizer with pressure set to 20,000-25,000 psi was used, and in addition, the suspensions were sonicated (Branson Digital sonifier 50/60 HZ) on ice with alternating 2 min cycle (15 pulses at 50% power with 30 s pauses on ice, until completing 2 min total sonication time). The lysed cells were centrifuged at 4 °C for 30 min at 11,000 × g. The precipitated fraction was re-suspended with 10 mL buffer D (buffer A plus 0.8% (V/V) Triton X-100, 0.1% sodium deoxycholate) and the suspension was sonicated to ensure the pellet is completely dissolved. This step repeated three times. Centrifugation was performed at 4 °C for 30 min at 11,000 × g. Finally, to solubilize IB, the pellet was suspended in 1 mL of buffer F (50 mM HEPES, pH 7.5, 8.0 M urea) per gram of precipitated fraction.
restriction site (underlined and italic) for cloning purposes. Anti-6xHis (bold) tag was used as a tag on the recombinant proteins to facilitate protein purification. gDNA of E. coli strain O157 was used as a template. The amplicon was ligated into NdeI/BamHI-digested pET11C plasmid as a component of a system for protein expression in E. coli yielding pET-his-hcaR. In this plasmid, T7 RNA polymerase (highly active constitutive promoter) was the promoter. Ampicillin was used as the selection marker. All recombinant plasmids were replicated in E. coli BL21 to have BL21 pET-his-hcaR. The correctness of cloning was confirmed by restriction enzyme digestion, and nucleotide sequence analysis of the insert.

Protein was purified as described previously (Luminy and Cedex 2011). Briefly, E. coli BL21 pET-his-hcaR was grown in 1 L of LB broth with 100 μg/mL ampicillin and 1 mM isopropyl-β-D-thiogalactoside (IPTG) at 37 °C overnight with shaking. The cells were harvested (1,700 x g, 10 min, 4 °C) and re-suspended in 25 mM lysis buffer (PBS, pH 7.5, 1 mM β-mercaptoethanol plus 1 tablet protease inhibitor Mini, EDTA-freetlyse). Bacterial cells were lysed using French pressure cell press (EmulsiFlex-C3 homogenizer) and stirred at 4 °C by adding Dnase, and whole proteins were purified. To get rid of aggregation, we kept the proteins on ice and then filtered it. HcaR protein purification was performed by AKTA FPLC system, which is a fully automated liquid chromatography system, where HcaR protein was purified using HiPrepTM HP 5 mL column. Before actual purification run, the Amber columns were charged, and the AKTA was equilibrated in AKTA Xpress program by AKTA Xpress. The purified proteins were collected from different tubes and the combined fractions were kept at 4 °C. Purified proteins were checked by running on the SDS gel.

In order to make polyclonal antibodies, several Swiss mice were immunized twice IP, in 10 days. The first injection was administered with a mixture of HcaR protein (50 μg per mouse) and complete Freund's adjuvant (Sigma) (1:1) on day 10, a booster injection (protein and incomplete Freund’s adjuvant (Sigma)) was given after titration of the antibody by enzyme-linked immunosorbent assay. Then from the serum, the total immunoglobulin Gs purified by protein A column (GE Healthcare) according to the manufacturer’s instructions.

Experimental animals. Female Swiss mice of 5 to 8 weeks with uniform weight (20 and 23 g) were used in this study (Harlan, The Netherlands). Mice were housed in plastic cages on softwood granules as bedding. The room was kept between 21 °C and 25 °C with 12/12 h light – dark cycles. The animals had free access to water and pelleted rodent food. In order to avoid stress-induced confounding factors mice were transferred to the lab 1 week before experimental manipulation.

In vivo toxicity test. The procedures used in the assays were approved by the local Animal Ethics Committee and conform to international standards of animal welfare (Approval P067/2015 of the Ethical Committee of KULeuven). A safe dose achieved from escalation experiment had no acute adverse effects upon IP administration. Three days after the last administration, animals were anesthetized and blood was collected using a standard retro-orbital puncture from each animal. Next, the mice sacrificed and underwent complete necropsy with gross examination and organ weights. The organs (heart, liver, spleen, kidney, bone marrow, brain, lung) were sampled and immersed in fixative in 4% paraformaldehyde. Formalin-fixed tissues after dehydration were routinely processed and embedded in paraffin blocks for histopathological examination. Five-micrometer-thick sections taken from these blocks (Thermo Scientific Microm HM355S microscope) were then stained with hematoxylin and eosin (Leica ST5010 Autostainer XL) and evaluated under a Leica DM 2500 light microscope by a board-certified veterinary pathologist. Hematological examination was performed using an automated high-resolution Hematology Analyzer (Coulter, USA). It was operated in positive ion mode (50 mm ammonium bicarbonate/ acetonitrile (ACN) (1:1) for 10 min at RT until the blue stain is gone and replacing the buffer by 100% ACN and incubating for 5 min. After the last cycle, the samples were dried by Speedvac and digested with 250 ng of modified trypsin (Promega) in 50 mM ammonium bicarbonate buffer (pH 8.3) overnight at 37 °C. Peptides were extracted by adding 5% TFA and 0.1% formic acid, and followed by 10% ACN + 0.1% formic acid (FA) and 90% ACN + 0.1% formic acid (FA) for 30 min. The extracted peptides were cleaned up using Pierce C18 spin Columns (Thermo Fisher Scientific) according to the manufacturer’s instructions. The samples were diluted in 10 μL with 5% ACN + 0.1% FA for injection in the Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, USA).

The urinary tract infection model. Male mice per cage on softwood granules as bedding. The room was equipped this strain with plasmid pCP20, resulting in the desired MG1655 strain.

Protein purification for MS analysis. For protein purification from SDS gels, the purified IBs were loaded on SDS gel (4–15% Mini-PROTEAN® TGX™ Precast Protein Gels, 10-well, 50 μl well volume) and stained with Coomassie blue (R250). The excised bands were cut by a sterile scalpel under a laminar flow. The gel slices were washed in several cycles by incubating them in 50 mM ammonium bicarbonate/ acetonitrile/water (8:2:1 v/v/v) for 10 min at RT. After three cycles, the gel was dehydrated with Speedvac and digested with 250 ng of modified trypsin (Promega) in 50 mM ammonium bicarbonate buffer (pH 8.3) overnight at 37 °C. The peptides were extracted by adding 5% TFA and 0.1% formic acid, and followed by 10% ACN + 0.1% formic acid (FA) and 90% ACN + 0.1% formic acid (FA) for 30 min. The extracted peptides were cleaned up using Pierce C18 spin Columns (Thermo Fisher Scientific) according to the manufacturer’s instructions. The samples were diluted in 10 μL with 5% ACN + 0.1% FA for injection in the Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, USA).
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Author contribution
F.R., J.S., and J.V.E. conceived the project. Lad.K., Lal.K., R.G., E.M., M.R., F.C., L.Y., and W.M. contributed to data collection. H.W., M.D., P.R.N., and P.H. contributed materials. LadK., Lal.K., N.L., R.G., A.A., S.C., R.R., F.R., and J.S. provided data analysis and interpretation. F.R. and J.S. drafted the article and Lad.K., Lal.K., R.G., N.K., E.M., R.R., M.R., F.C., L.Y., M.S., H.W., M.D., W.M., P.R.N., P.H., A.A., S.C., J.V.E., F.R., and J.S. provided critical feedback and revisions.

Additional information
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Competing interests: J.S. and F.R. are listed as inventors of patents held by their hold institution VIB, covering the peptides described in this manuscript, and which are licenced to Aelin Therapeutics (Leuven, Belgium), of which J.S. and F.R. are the scientific founders. All remaining authors declare no competing financial interests.

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