Supplementary Information

Supplementary Methods

Minimal Inhibitory Concentration (MIC) Determination

Minimal inhibitory concentrations were determined by the agar dilution method on isosensitfest agar according to British Society for Antimicrobial Chemotherapy guidelines (1), except that dilution series were carried out at concentration intervals of 0.1 mg/L (nalidixic acid) and 0.001 mg/L (other antibiotics).

AhQnr Expression and Purification

The ahqnr open reading frame was amplified by the polymerase chain reaction (5’ 94°C followed by 30 cycles of 1’ 95°C, 1’ 55°C, 1’ 72°C) using primers (Table S1) AhQnrFwd and AhQnrRev (Sigma-Genosys, Poole, UK) and RedTaq DNA polymerase (Sigma). The resulting PCR product (~650 bp) was purified on an agarose gel and cloned into the Nde1 and Xho1 sites of the T7 expression vector pET-26b to generate plasmid AhQnr:pET-26b. The integrity of the cloned insert was verified by DNA sequencing (The Sequencing Service, University of Dundee, U.K.). Plasmid AhQnr:pET-26b was transformed into the E. coli strains TOP 10 (Invitrogen) for propagation and BL21 (DE3) (Novagen) for protein expression.
E. coli BL21 (DE3) containing AhQnr:pET-26b was grown in LB media supplemented with 50 μg/ml kanamycin until the optical density at 600 nm reached approximately 0.5. AhQnr expression was induced by addition of 1 mM IPTG and cells grown for a further 3 – 4 hours at 37°C. Cells were harvested by centrifugation (4°C, 5 000 g). Lysate was prepared by resuspending the pellet in Buffer A (20 mM Tris-HCl pH 8.5, 5 mM β-mercaptoanol, 10% glycerol), passage through a One Shot microfluidiser (Constant Cell Disruption Systems, Daventry, UK) and centrifugation (15 000 g, 25 minutes). The cleared lysate was applied to a 30 ml Q-sepharose FF column (GE Healthcare), the column was washed with buffer A until the absorbance at 280 nm returned to baseline and bound proteins eluted with a 1 L 0 – 1 M NaCl gradient. Fractions containing AhQnr were identified by SDS-PAGE, pooled and dialysed overnight against Buffer A. After centrifugation (15 000 g, 25 minutes) the dialysate was applied to a 50 ml heparin-sepharose column (GE Healthcare) and bound proteins eluted on a 500 ml 0 – 0.3 M NaCl gradient. This step was omitted for material used to produce the orthorhombic crystal form. Fractions containing AhQnr were pooled, concentrated by centrifugation (Amicon Ultra-15) to be further purified by size exclusion chromatography on a 350 ml Superdex S-75 column (GE Healthcare) in Buffer B (20 mM Tris-HCl pH 8.0, 1M NaCl, 5mM β-mercaptoethanol, 5% glycerol; protein for crystallization, biophysical and activity assays) or a 350 ml Superdex S-200 HR column (GE Healthcare) in Buffer C (20 mM HEPES pH 8.0, 1 M NaCl, 5% glycerol; protein for reductive methylation).

Selenomethionine-substituted AhQnr was expressed in E. coli B834 (DE3) (Novagen) harbouring plasmid AhQnr:pET-26b, grown in SelenoMethionine Expression Media (Molecular Dimensions) following the manufacturer’s protocol. Briefly, a 100 ml bacterial culture was grown overnight at 37 °C in LB broth, cells were pelleted by centrifugation (20
minutes, 4 000 g), washed 3 times in 100 ml of sterile water, resuspended in 1 ml of water and inoculated into 1 L of prewarmed (30° C) SelenoMethionine Expression Media containing L-SeMet. Cells were grown for 2 h before the addition of IPTG to 1 mM and growth was continued for a further 6 hours prior to cell harvest. Expressed protein was purified as described above.

**Crystallisation**

Wild-type AhQnr dialyzed into Buffer E (20 mM phosphate pH 8.0, 100 mM KCl, 5% glycerol, 20 mM DTT) and concentrated to 10 mg/ml crystallized from 0.1 M sodium cacodylate pH 6.8, 1.5 M sodium acetate, 20 mM DTT using hanging drop vapour diffusion at 18 °C mixing 1 μl of protein and 1 μl of precipitant solution. A single crystal produced by this method (orthorhombic crystal form; Table 1) diffracted to 2.2 Å resolution but proved impossible to reproduce despite extensive efforts.

Accordingly, we used reductive methylation of surface lysine residues (2) to search for new crystal forms of improved reproducibility. AhQnr in Buffer C at 4 mg/ml was reacted with 1 M dimethylamine borane complex (DMAB) solution (20 μl/ml protein) and 1 M formaldehyde (40 μl/ml protein) for 2 hours at 4 °C in the dark with shaking at 100 rpm. This procedure was repeated 4 times, followed by a final addition of DMAB (10 μl/ml protein). The mixture was incubated for a further 12 hours and unused reactants removed by gel filtration (Superdex S-200 HR as above) in Buffer F (20 mM Tris-HCl pH 8.0, 50mM NaCl, 10mM DTT, 5% glycerol). Reductively methylated AhQnr in Buffer F was concentrated to 5 mg/ml and fresh DTT added to a final concentration of 10 mM. After 10 h incubation on ice, hanging drop crystallisation experiments were set up using 1.5 μl DTT-treated methylated AhQnr, 1.5 μl precipitant (0.1 M bis-tris propane pH 7.5, 0.2 M potassium thiocyanate, 16 – 20% PEG 3350, 8 – 16% glycerol,
40 mM DTT) and 0.3 μl 200 mM spermine-HCl per drop. Drops were equilibrated over 500 μl of precipitant solution at 18 °C. Crystals (monoclinic crystal form, Table 1) normally appeared from light precipitates within 24 hours and grew to their maximal size in 15 – 20 days. SeMet AhQnr (2.5 mg/ml) was methylated and crystallized as above, with the best crystals obtained at concentrations of 14 – 16% PEG 3350 and 8 – 12% glycerol.

**Structure Solution**

AhQnr crystals were mounted in rayon or mylar loops (Molecular Dimensions), cryoprotected by transient exposure to precipitant solution plus 20 % DMSO (orthorhombic crystal form) or 30 – 35 % glycerol (monoclinic crystal form) and snap-frozen in liquid nitrogen for subsequent X-ray data collection. Diffraction data were collected on beamline 10.1 (3) of the U.K. synchrotron radiation source, (SRS) Daresbury (orthorhombic crystal form) and beamlines I03 (monoclinic crystal form, native data set) and I02 (SeMet data set) of the U.K. Diamond Light Source. For the Se-Met data set the wavelength for data collection with maximal anomalous scattering (i.e. maximal f”) was selected based on analysis of an X-ray fluorescence scan of a mounted crystal using the program CHOOCH (4). Diffraction data were indexed and scaled using the programs MOSFLM (5) and SCALA (6). Data processing statistics are given in Table 1.

The AhQnr structure was solved by single-wavelength anomalous diffraction (SAD; (7)) using data collected from a single crystal of the Se-Met protein. Automated Patterson searches using SOLVE (8) as implemented in PHENIX (9), readily located four Se sites in the monoclinic asymmetric unit. Electron density maps calculated using these initial phases at 3.0 Å resolution were, after solvent flattening and density modification in RESOLVE (10),
sufficient to enable a partial polyserine model, comprising 4 pentapeptide repeat coils originally derived from the MfpA structure (11), to be placed for one of the two subunits present in the asymmetric unit using MOLREP (12), as implemented in the CCP4 suite (13). Application of NCS (non-crystallographic symmetry) operators derived from the Se positions, together with multiple rounds of manual (Coot; (14)) and automated (RESOLVE) rebuilding and phase extension against the 2.0 Å native data set, permitted us to construct an improved model comprising 235 of the total 432 residues of the AhQnr dimer. At this point re-running RESOLVE, starting from the original SOLVE phases, imposing NCS operators derived from this model and including the 2.0 Å native data set, resulted in dramatically improved electron density maps. Subsequent rounds of manual rebuilding in Coot with addition of water using ARP (15) and refinement with REFMAC5 (16) proceeded smoothly. The resulting structure, after removal of the projecting loops 1 and 2, was then used to solve that of the orthorhombic crystal form by molecular replacement using Phaser (17). Refinement statistics for the two structures are given in Table 1.

Generation and Production of AhQnr Mutants

Mutants were constructed in a hexahistidine-tagged variant of AhQnr, AhQnr-6His, in order to facilitate protein purification. AhQnr-6His was generated by using primers AhQnrFwd and AhQnrRevNoStop (Table S1) to amplify the AhQnr open reading frame without the C-terminal stop codon and cloning this DNA fragment into the Nde1 and Xho1 sites of the pET-26b expression vector. This created a plasmid, AhQnr-6His:pET-26b, expressing AhQnr fused to a C-terminal hexahistidine tag.

Deletion mutants of AhQnr were constructed on the AhQnr-6His:pET-26b template using the QuikChange II mutagenesis kit (Agilent) according to the manufacturer’s instructions with
primers as specified (Table S1). Amplifications were performed for 1’ at 95°C followed by 25 cycles of 1’ 95°C, 2’ 55°C, 8’ 68°C. The double deletion mutant (Δdouble) was constructed in a second round of mutagenesis where loop1 was deleted from Δloop2. The presence of the expected mutations was confirmed by complete sequencing in both directions of the AhQnr open reading frame. AhQnr-6His and Δloop2 were expressed as described for wild-type AhQnr (above). Δloop1 and Δdouble were expressed in *E. coli* ArcticExpress (DE3) (Agilent) induced with 1 mM IPTG at an OD600 of 0.5 for 48 hours at 12 °C.

AhQnr-6His and mutant proteins were purified by two column chromatography steps carried out at 4°C. Clarified cell lysates were prepared as above in Buffer G (40 mM phosphate pH 8.0, 20 mM imidazole, 300 mM NaCl, 10% glycerol), applied to a 5 ml Ni-NTA Superflow column (Qiagen, Crawley, UK) and recirculated for at least 1 hour. The column was washed extensively in Buffer G and bound proteins eluted on a step gradient using 50 ml washes in Buffer G containing increasing concentrations of imidazole (50 mM, 100 mM, 250 mM, 500 mM). Eluted wild-type and Δloop2 proteins were concentrated by centrifugation and further purified on a 350 ml Superdex S-200 HR column equilibrated in Buffer H (25 mM Tris-HCl pH 7.5, 2 mM DTT, 5% glycerol). Both the Δloop1 and Δdouble mutants precipitated in Buffer G in the presence of salts (NaCl and imidazole); these precipitates were collected by centrifugation and resolubilised in Buffer H prior to size exclusion chromatography.
Supplementary Figure Legends

Figure S1. Alignment of Qnr Family Members from Gram-negative Bacteria. Figure shows 61 Qnr family members aligned using ClustalW (18) as implemented in the program Geneious (Biomatters Ltd.). Features of the AhQnr structure (coils 1-9 and loops 1 and 2) are annotated upon the AhQnr sequence (*Aeromonas hydrophila* ATCC 7966). Invariant amino acids are highlighted in red, conserved amino acids (≥ 80 % similarity according to the Blosum85 scoring matrix) are highlighted in blue. The consensus sequence (top) is numbered according to the AhQnr sequence.

Figure S2. Phylogenetic Tree of Qnr and Related Proteins.
Phylogenetic tree of 81 PRP sequences (61 as above plus 20 additional sequences from Gram-positive bacteria) aligned as above.

Figure S3. Predicted Properties of Qnr Family Members from Gram-negative Bacteria.
Predicted solubility (blue; calculated according to (19)); and charge at pH 7.0 (green) and pI (cyan) (calculated using the program Protean (DNASTAR, Inc.)) for Qnr family members from Gram-negative bacteria.

Figure S4. Architecture and Sequence of Pentapeptide Repeats and Projecting Loops in AhQnr Structure. A. Cross section of AhQnr pentapeptide repeat (coil 4; chain B) viewed down the four fold helical axis of the β-helix. Electron density shown is $|2F_o| - |F_c| \cdot \phi_{calc}$, contoured at 1.5σ. Residue positions in each pentapeptide repeat are labeled from $i-2$ to $i+2$, where $i$ is the central hydrophobic position in each repeating unit, as previously described for
other PRP structures (20-23). C. Superposition of AhQnr structures showing variable conformations of projecting loops. Chains A and B of the monoclinic (P2_1) structure are coloured green and cyan; chains A and B of the orthorhombic (P2_12_12) structure are coloured purple and red.

**Figure S5. Loop Deletions do not Affect AhQnr Structure.** A. Far-UV CD spectra of AhQnr and mutants. Protein concentrations: AhQnr 21.1 μM; AhQnr-6His 31.7 μM; Δloop1 31.3 μM; Δloop2 30.0 μM; Δdouble 31.6 μM. AhQnr spectrum was acquired in Buffer D. All other spectra were acquired in Buffer H. B. Size exclusion chromatography (Superdex S-200 HR) of AhQnr and mutants. Absorbance readings (280nm) are normalized to the height of the AhQnr elution peak for each individual trace (y-axis). Labels show elution positions for size marker proteins (Sigma) carbonic anhydrase (CA; 29.0 kDa), bovine serum albumin (BSA, 66.2 kDa) and β-amylase (β-A; 200 kDa). C. Plot of log_{10} molecular mass versus elution volume for AhQnr and mutants derived from data shown in Figure S5B. Straight line shows fit to data for calibration proteins (labels as above plus cytochrome c (CytC; 12.4 kDa)). Elution positions are shown for AhQnr (dimer molecular mass 48 473 Da), AhQnr-6His (50 603 Da), Δloop1 (49 113 Da), Δloop2 (48 234 Da) and Δdouble (46 745 Da). Elution positions relative to standards are consistent with all AhQnr samples existing as dimers in solution.

**Figure S6. AhQnr and its Deletion Mutants do not Inhibit Supercoiling Activity of DNA Gyrase.** ATP-dependent DNA supercoiling activity of DNA gyrase assayed in the presence of AhQnr-6His and mutants. Panel A. AhQnr-6His; B. Δloop1; C. Δloop2; D. Δdouble. Lanes: N, negative control (relaxed pBR322 only; 500ng); P, positive control (relaxed pBR322 plus
DNA gyrase (1U)); 1 – 40, relaxed pBR322 plus DNA gyrase (1U) with AhQnr or mutants (concentration added in μM). Positions of migration for relaxed and supercoiled pBR322 DNA are labeled as R and S respectively.
Supplementary References

1. Andrews, J.A. (2001) Determination of minimum inhibitory concentrations. *J Antimicrob Chemother.*, **48**, Supplement 1, 5-16.

2. Walter, T.S., Meier, C., Assenberg, R., Au, K.-F., Ren, J., Verma, A., Nettleship, J.E., Owens, R.J., Stuart, D.I. and Grimes, J.M. (2006) Lysine Methylation as a Routine Rescue Strategy for Protein Crystallization. *Structure*, **14**, 1617-1622.

3. Cianci, M., Antonyuk, S., Bliss, N., Bailey, M.W., Buffey, S.G., Cheung, K.C., Clarke, J.A., Derbyshire, G.E., Ellis, M.J., Enderby, M.J. *et al.* (2005) A high-throughput structural biology/proteomics beamline at the SRS on a new multipole wiggler. *J Synchrotron Radiat*, **12**, 455-466.

4. Evans, G. and Pettifer, R.F. (2001) CH OOCH: a program for deriving anomalous-scattering factors from X-ray fluorescence spectra. *J Appl Cryst.*, **34**, 82-86.

5. Leslie, A.G.W. (1992) Recent changes to the MOSFLM package for processing film and image plate data *Joint CCP4 + ESF-EAMCB Newsletter on Protein Crystallography*, **26**, 27-33.

6. Evans, P.R. (2005) Scaling and assessment of data quality. *Acta Crystallogr D Biol Crystallogr.*, **62**, 72-82.

7. Dauter, Z., Dauter, M. and Dodson, E. (2002) Jolly SAD. *Acta Crystallogr D Biol Crystallogr.*, **58**, 494-506.

8. Terwilliger, T.C. and Berendzen, J. (1999) Automated MAD and MIR structure solution. *Acta Crystallogr D Biol Crystallogr.*, **55**, 849-861.

9. Adams, P.D., Afonine, P.V., Bunkoczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, L.W., Kapral, G.J., Grosse-Kunstleve, R.W. *et al.* (2010) PHENIX: a
comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr.*, **66**, 213-221.

10. Terwilliger, T.C. (2000) Maximum likelihood density modification. *Acta Crystallogr D Biol Crystallogr*, **56**, 965-972.

11. Hegde, S.S., Vetting, M.W., Roderick, S.L., Mitchenall, L.A., Maxwell, A., Takiff, H.E. and Blanchard, J.S. (2005) A fluoroquinolone resistance protein from Mycobacterium tuberculosis that mimics DNA. *Science*, **308**, 1480-1483.

12. Vagin, A. and Teplyakov, A. (2010) Molecular replacement with MOLREP. *Acta Crystallogr D Biol Crystallogr*, **66**, 22-25.

13. Collaborative Computational Project No 4. (1994) The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr*, **50**, 760-763.

14. Emsley, P. and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr*, **60**, 2126-2132.

15. Lamzin, V.S. and Wilson, K.S. (1997) Automated refinement for protein crystallography. *Methods Enzymol*, **277**, 269-305.

16. Murshudov, G.N., Vagin, A.A. and Dodson, E.J. (1997) Refinement of Macromolecular Structures by the Maximum-Likelihood Method. *Acta Crystallogr D Biol Crystallogr*, **53**, 240-255.

17. McCoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Storoni, L.C. and Read, R.J. (2007) Phaser crystallographic software. *J Appl Cryst.*, **40**, 658-674.

18. Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R. et al. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics*, **23**, 2947-2948.

19. Wilkinson, D.L. and Harrison, R.G. (1991) Predicting the solubility of recombinant proteins in Escherichia coli. *Biotechnology (N Y)*, **9**, 443-448.
20. Buchko, G.W., Ni, S., Robinson, H., Welsh, E.A., Pakrasi, H.B. and Kennedy, M.A. (2006) Characterization of two potentially universal turn motifs that shape the repeated five-residues fold--crystal structure of a lumenal pentapeptide repeat protein from Cyanothece 51142. *Protein Sci*, **15**, 2579-2595.

21. Buchko, G.W., Robinson, H., Pakrasi, H.B. and Kennedy, M.A. (2008) Insights into the structural variation between pentapeptide repeat proteins--crystal structure of Rfr23 from Cyanothece 51142. *J Struct Biol.*, **162**, 184-192.

22. Vetting, M.W., Hegde, S.S., Fajardo, J.E., Fiser, A., Roderick, S.L., Takiff, H.E. and Blanchard, J.S. (2006) Pentapeptide repeat proteins. *Biochemistry*, **45**, 1-10.

23. Ni, S., Sheldrick, G.M., Benning, M.M. and Kennedy, M.A. (2009) The 2A resolution crystal structure of HetL, a pentapeptide repeat protein involved in regulation of heterocyst differentiation in the cyanobacterium Nostoc sp. strain PCC 7120. *J Struct Biol.*, **165**, 47-52.
Supplementary Tables

Table S1: Oligonucleotide Primers. Underlined sequences indicate NdeI restriction site for AhQnrFwd primer and XhoI restriction sites for AhQnrRev and AhQnrRevNoStop primers.

| Primer               | Sequence                                                                 |
|----------------------|--------------------------------------------------------------------------|
| AhQnrFwd             | 5'-ATTATT CATATG ATCAAGGACAAGTGCTTCG-3                                   |
| AhQnrRev             | 5'-ATT [UNDERLINED] CTCGAG TCA GGGGAAGACGATCAGC-3'                       |
| AhQnrRevNoStop       | 5'-ATT [UNDERLINED] CTCGAG GGGGAAGACGATCAGCC-3'                         |
| Δloop1sense          | 5'-TCGTCGACTGCAGCTTCTATAGCTGTGTGTTCGTCG-3'                              |
| Δloop1antisense      | 5'-GCAGCAACACAGCTATAGAAGCTGCAGTCGACGA-3'                                |
| Δloop2sense          | 5'-GGGCCAGCTTTGCGGAGGCATCTGAC-3'                                       |
| Δloop2antisense      | 5'-GTCAGATGGGCCTCGGCAAAGCTGGCCC-3'                                     |

Table S2: Quinolone MICs for AhQnr-expressing *E. coli* BL21 (DE3).

| Quinolone          | AhQnr:pET-26b MIC (mg/L) | Empty pET-26b control MIC (mg/L) |
|--------------------|--------------------------|----------------------------------|
| Nalidixic acid     | 0.9                      | 0.5                              |
| Norfloxacin        | 0.016                    | 0.007                            |
| Levofloxacin       | 0.007                    | 0.004                            |
| Ciprofloxacin      | 0.004                    | 0.001                            |
Supplementary Figures

Figure S1
Figure S2
Figure S3
Figure S5

**A.**

![Graph showing mean residue ellipticity vs. wavelength (nm).](image)

**B.**

![Graph showing peak height vs. elution volume (ml).](image)

**C.**

![Graph showing log10 MW vs. elution volume (ml).](image)
Figure S6

A. [AhQnr-6His] (µM)

B. [Δloop1] (µM)

C. [Δloop2] (µM)

D. [Δdouble] (µM)