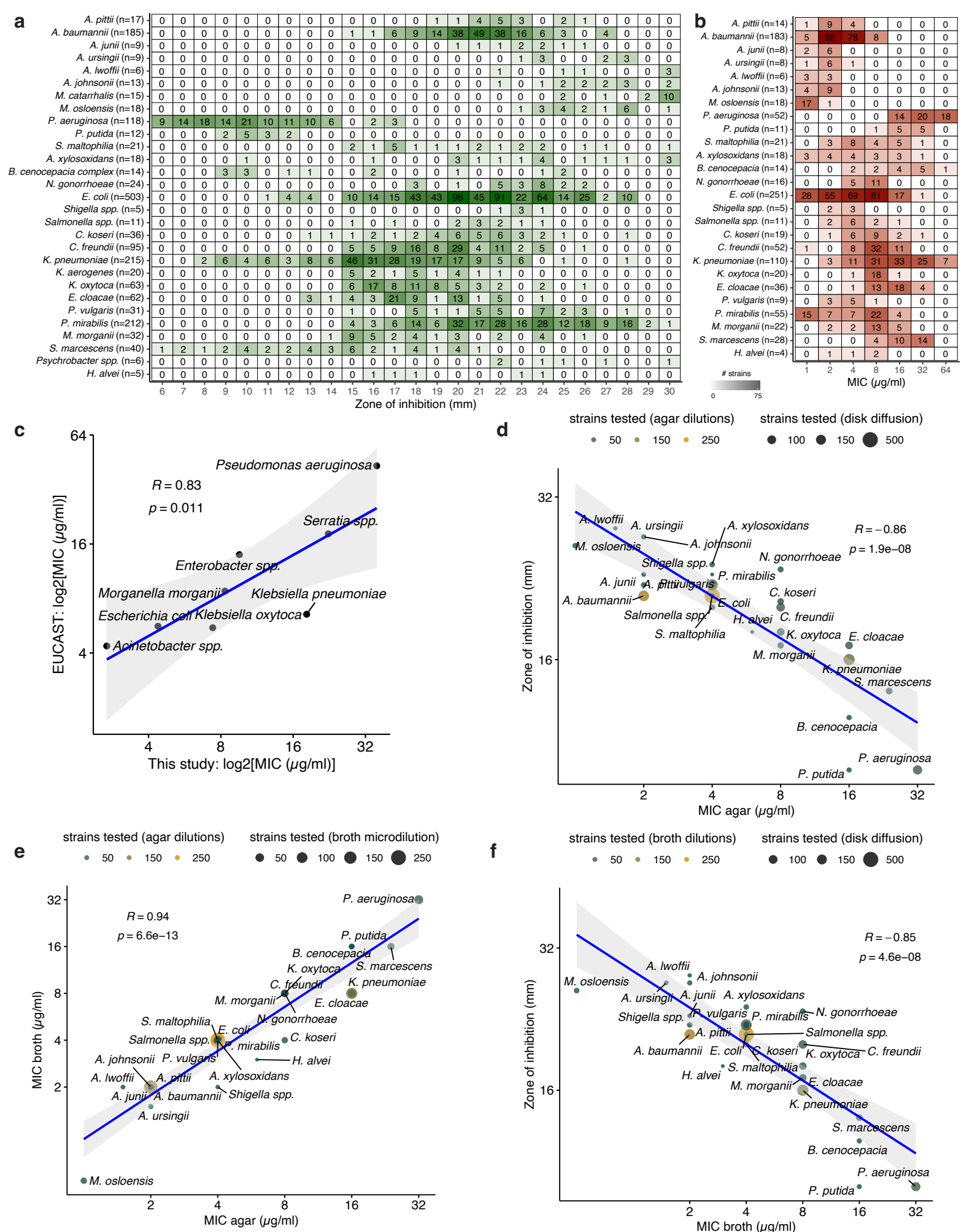
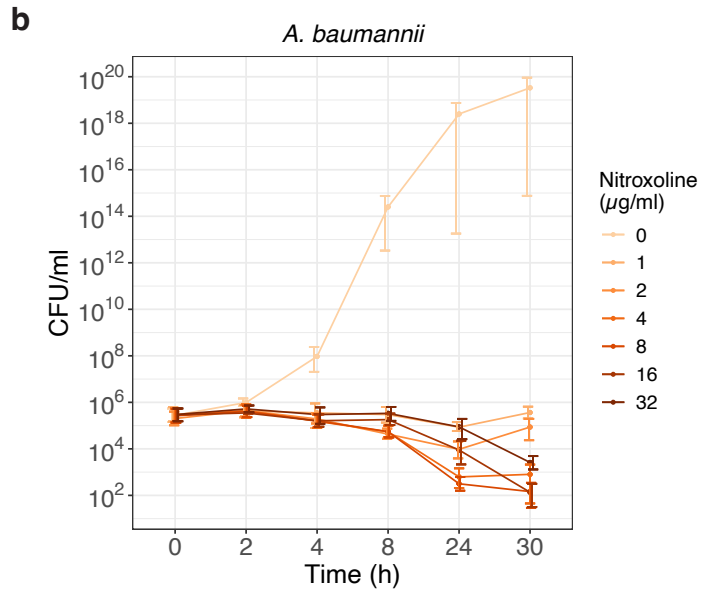
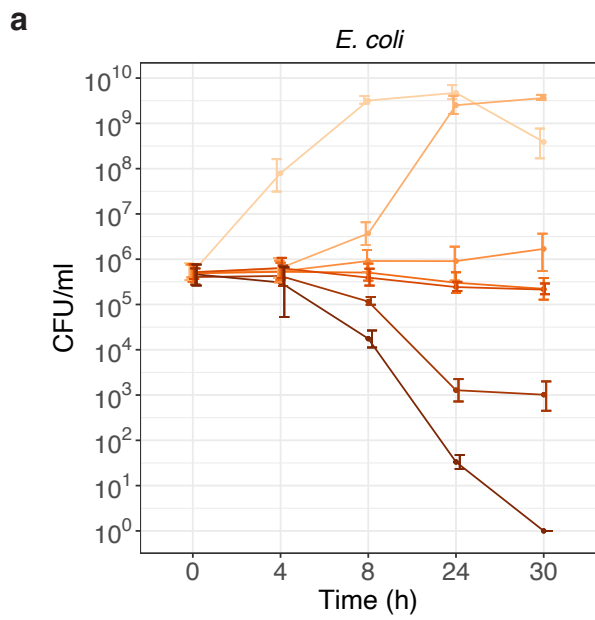
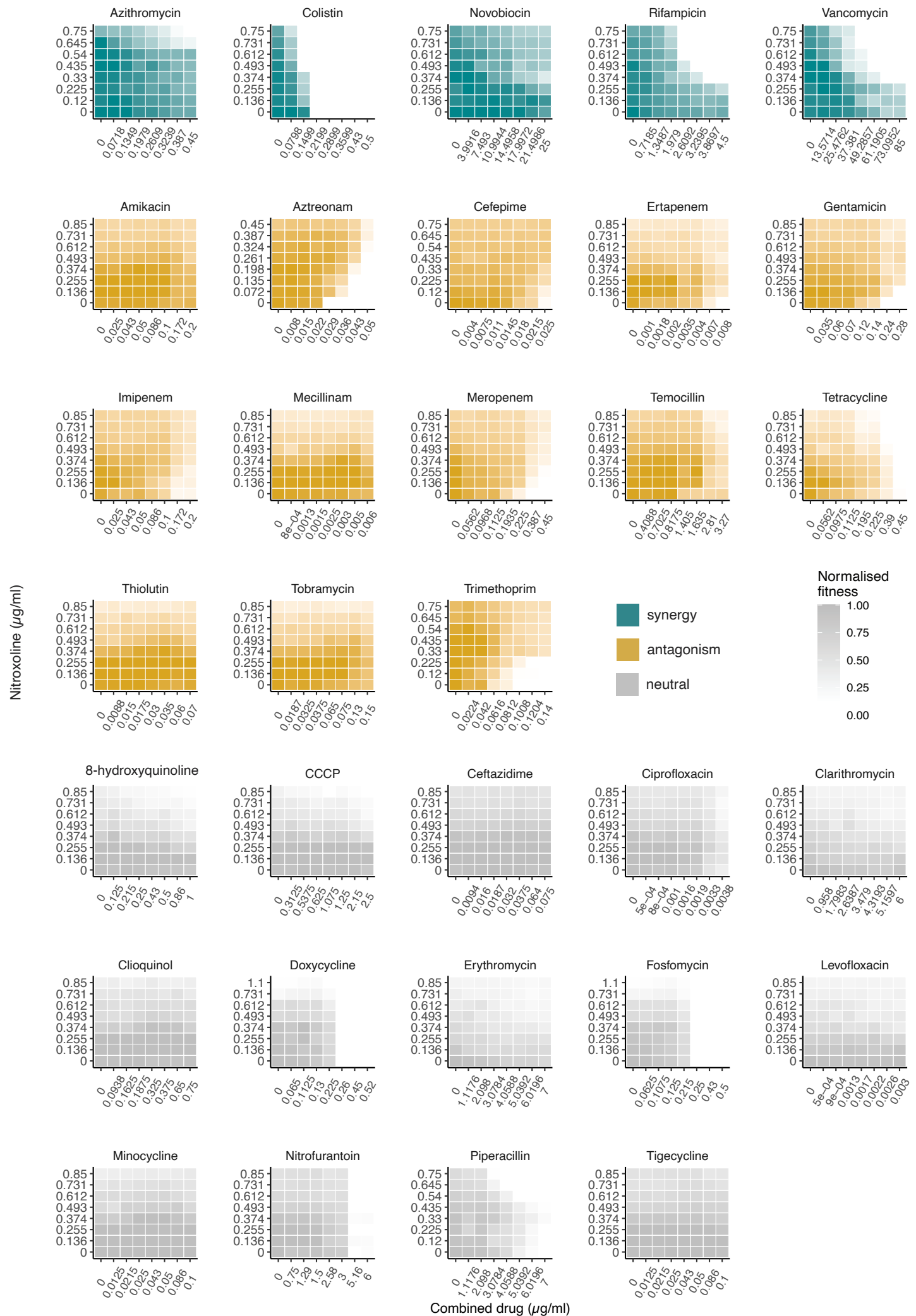




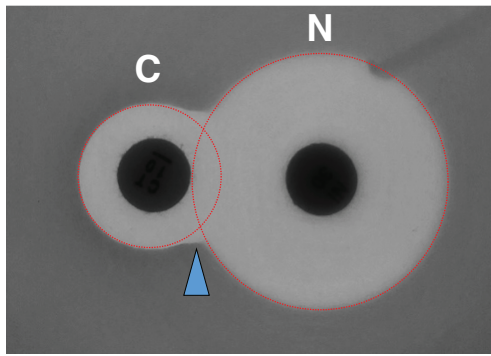
Supplementary Fig. 1. Nitroxoline MIC measurement via disk diffusion and agar dilutions and agreement across the different assays and with EUCAST. a-b. MIC measurements via disk diffusion (a) and agar dilution (b). Sensitivity is expressed as inhibition zone diameter (mm) (a) or μg/ml (b) (Methods). Each value is annotated and color-coded according to the number of strains it was measured in. Multiple species are aggregated for *Salmonella* spp., *Shigella* spp., *Burkholderia cenocepacia* complex and *Psychrobacter* spp. (Source Data). **c.** Concordance between MICs measured in broth dilution in this study and according to EUCAST¹⁹ for the eight overlapping species. **d-f.** Concordance between MICs measured across three orthogonal methods: agar dilution and disk diffusion (d), broth dilution and disk diffusion (e), broth dilution and disk diffusion (f). Pearson's correlation coefficient and p-value are shown. Disk diffusion results are expressed as in Supplementary Fig. 1a.



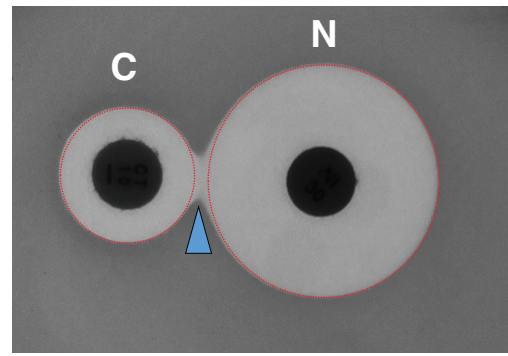
Supplementary Fig. 2. Bactericidal activity of nitroxoline. **a.** Nitroxoline is bacteriostatic against *E. coli* BW25113 at concentrations up to 4 x MIC (16 $\mu\text{g/ml}$) and bactericidal from 8 x MIC (32 $\mu\text{g/ml}$). Results are represented as in Fig. 1e. **b.** Time-kill curves for nitroxoline in *A. baumannii* ATCC 19606T (Fig. 1e) including the no-drug control. Results are represented as in Fig. 1e.



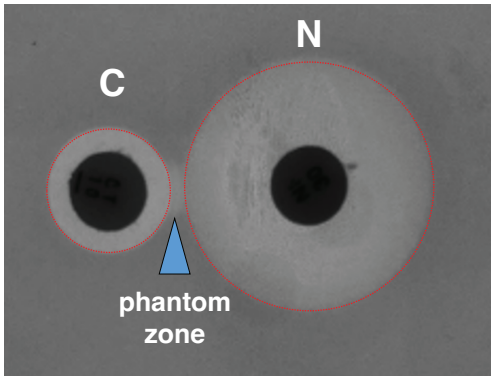
A. baumannii isolate 1



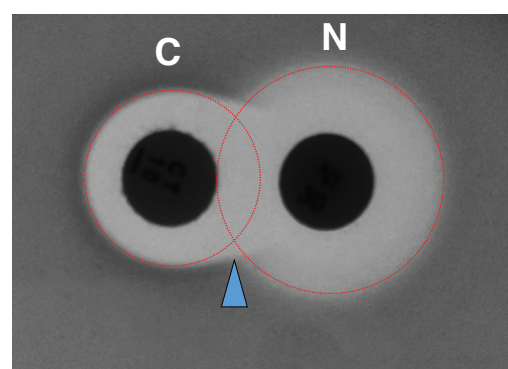
A. baumannii isolate 2



E. coli NCTC13846

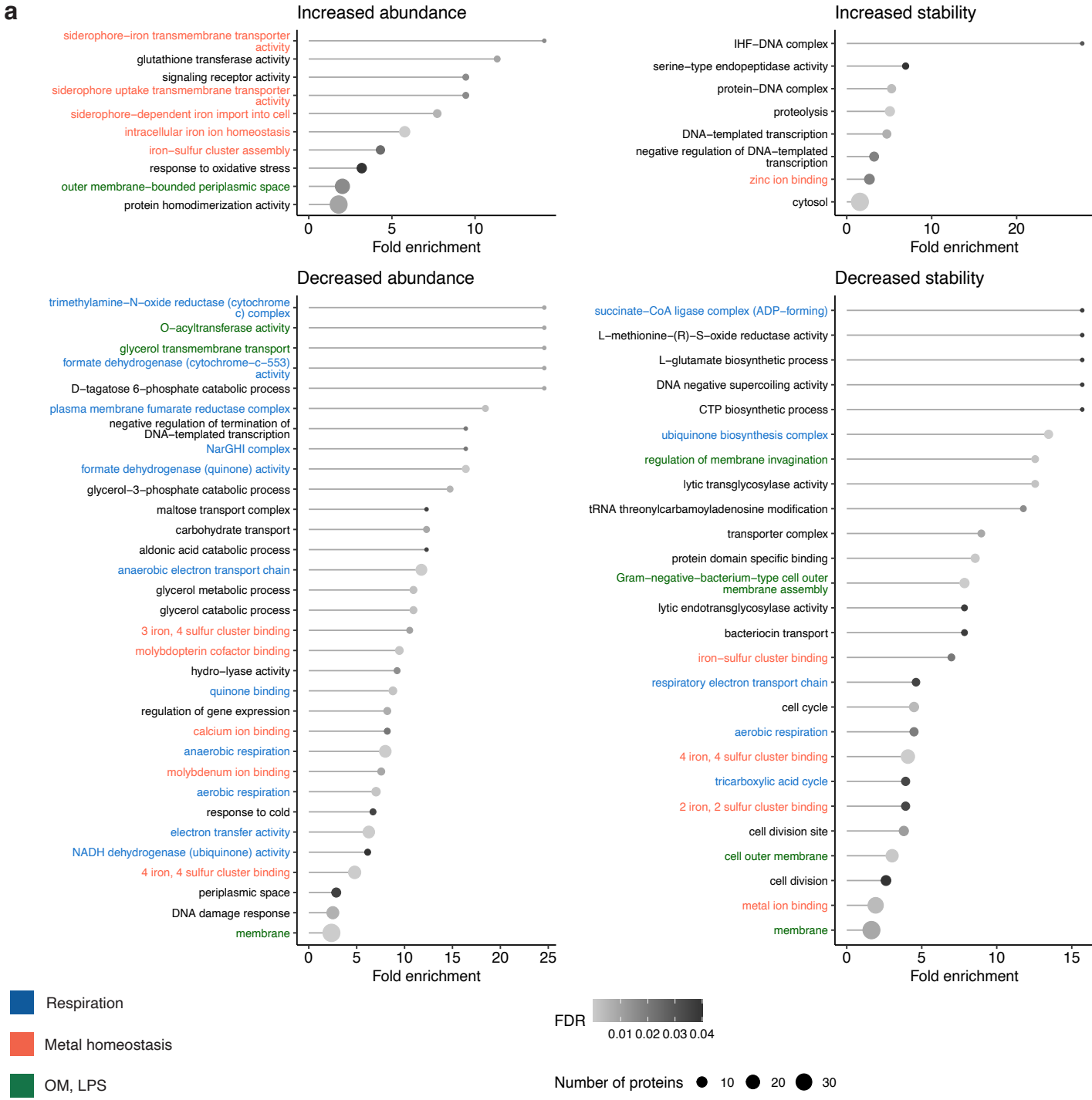


K. pneumoniae BAA-1705

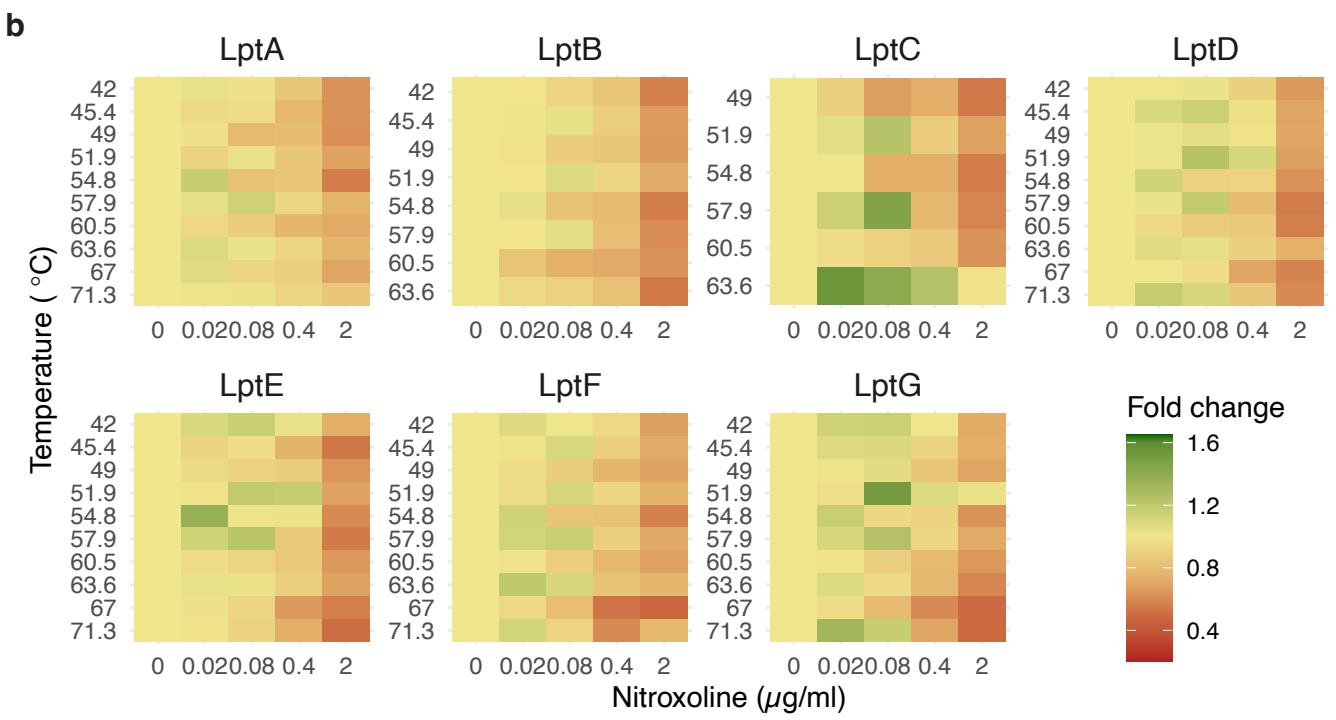


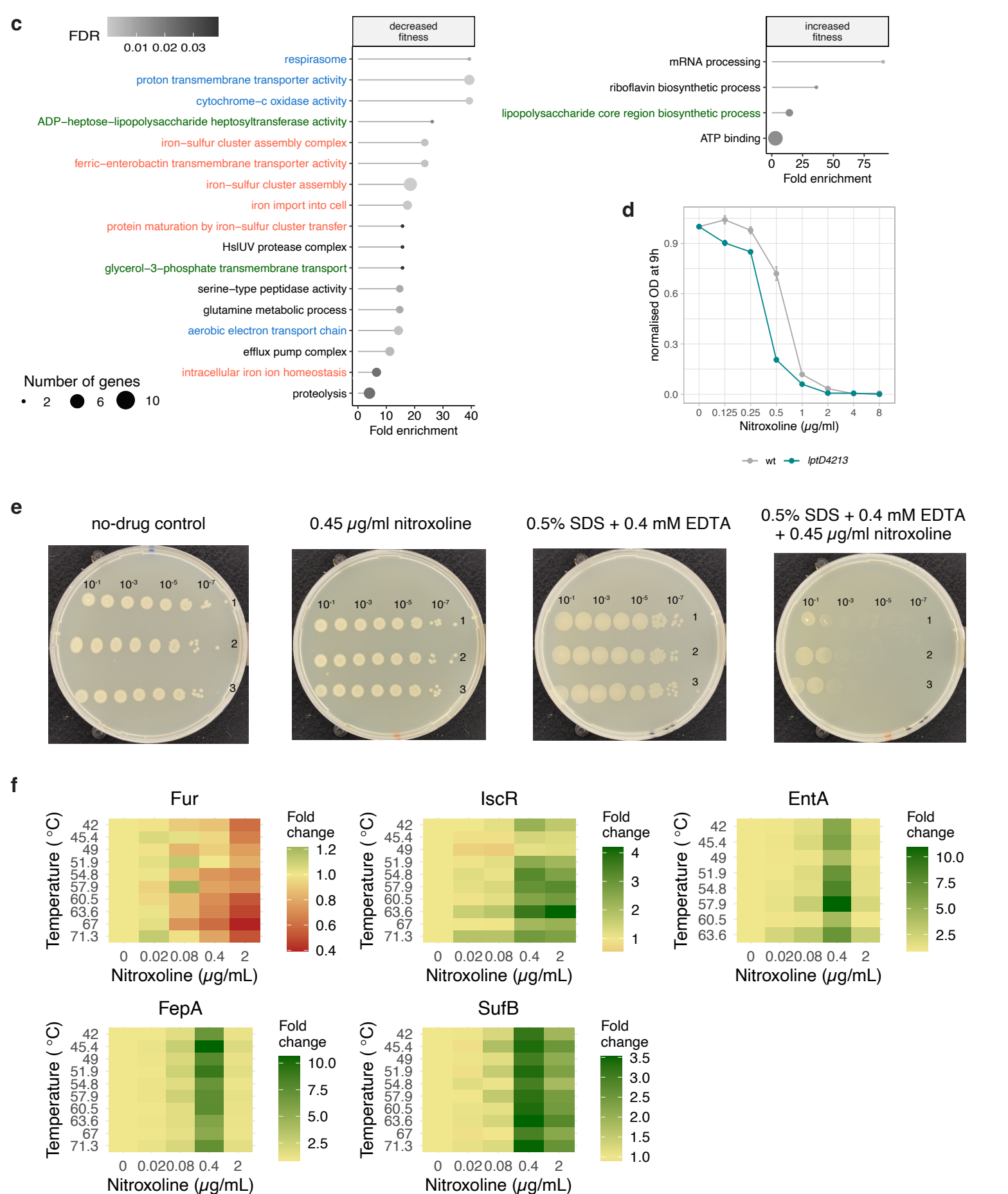
Supplementary Fig. 4. Colistin-nitroxoline synergy across Gram-negative species (disk diffusion). A nitroxoline disk (N; 30 μ g, Liofilchem) and a colistin disk (C; μ g, Oxoid) were placed on a Mueller-Hinton agar plate (Oxoid), inoculated with the indicated isolate. The red dotted line indicates the zone of inhibition (ZOI), the blue arrow the synergism as widened circular ZOI or additional growth inhibition outside the ZOI (phantom zone). Interactions are evident despite the large molecular weight of colistin, which prevents effective diffusion into the agar.

a

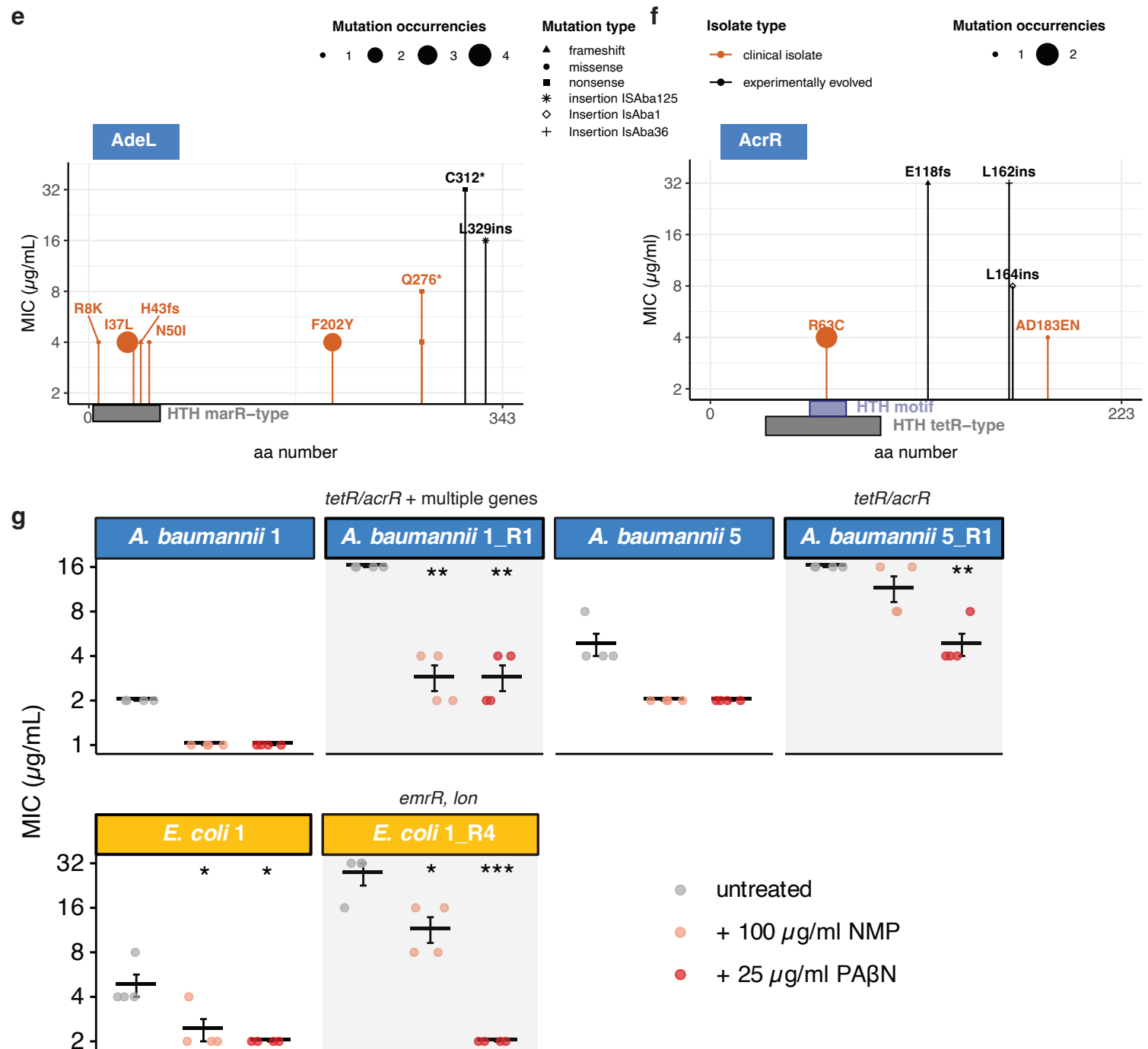


b





Supplementary Fig. 5 (continued). Nitroxoline perturbs the OM and affects metal homeostasis in *E. coli*. **a.** GO enrichment of 2D-TPP abundance and stability changes (Fig. 3a, Supplementary Data 3). Significantly enriched GO terms are shown (adjusted p-value < 0.05, Fisher's exact test). Dot size represents the number of hit proteins for each term, dot colour the adjusted p-value, label colour highlights GO terms related to OM or LPS, metal homeostasis, and respiration. **b.** Nitroxoline affects the abundance and stability of the LPS transport machinery. In the thermal stability profiles of Lpt system members, protein fold change is shown for each temperature and nitroxoline concentration. **c.** GO enrichment of chemical genetics hits (Fig. 3b, Supplementary Data 4). Results are represented as in Supplementary Fig. 5a. **d.** Nitroxoline is more potent upon genetic perturbation of the OM. Nitroxoline susceptibility in *E. coli* BW25113 and its OM-defective derivative strain, carrying the *lptD4213* mutation⁴⁴. Data is shown as in Fig. 2b. For full growth curves see https://github.com/ElisabettaCacace/nitroxoline_2024/blob/main/figures/additional_figures.pdf. **e.** Lower concentrations of nitroxoline and EDTA were tested than in Fig. 3d (EOP assay, Methods) to show the first dosage at which growth was visible upon combination. **f.** Nitroxoline induces effects in 2D-TPP consistent with iron-sulfur cluster disruption. Thermal profiles are represented as in Supplementary Fig. 5b.

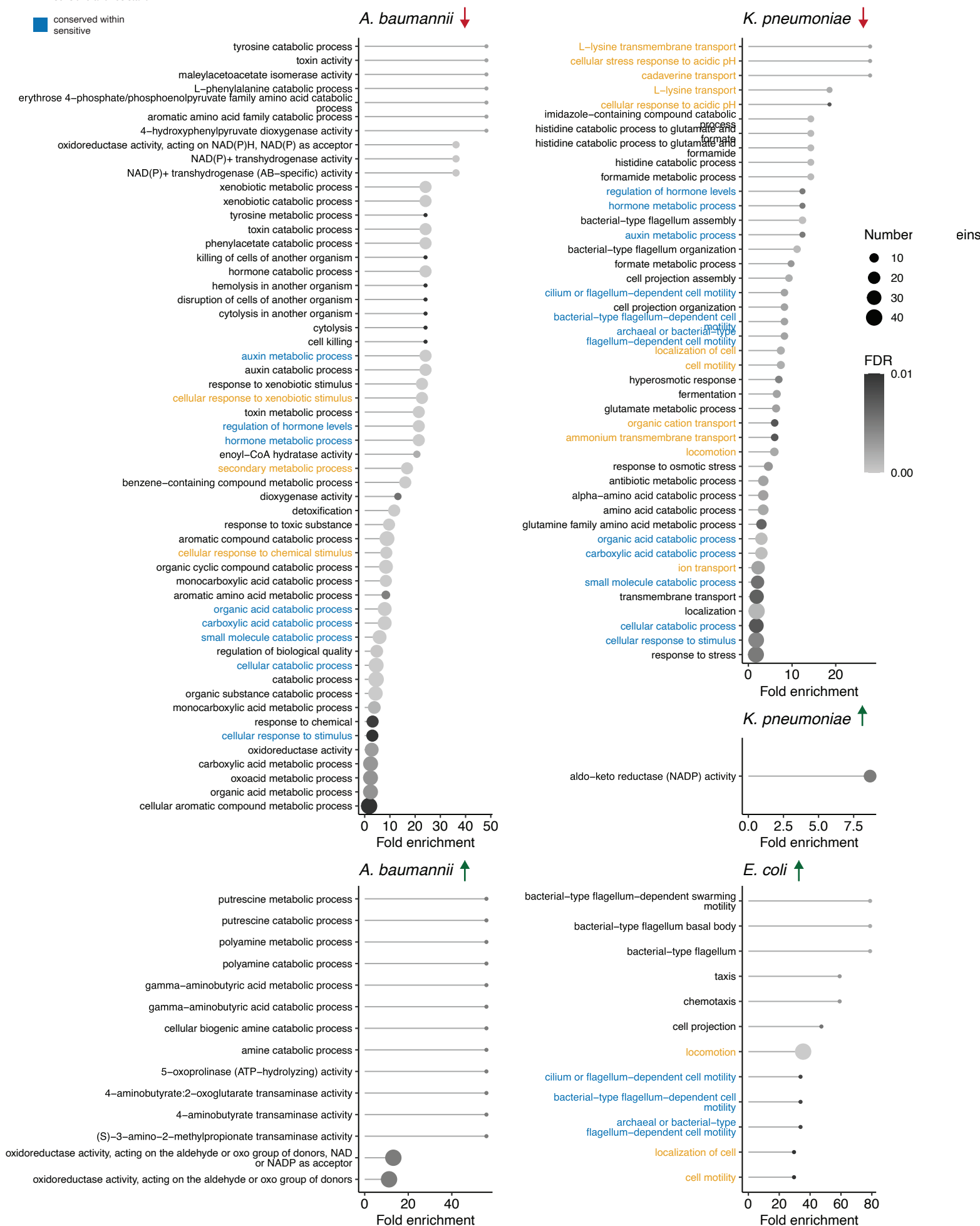


Supplementary Fig. 6. Nitroxoline resistance is based on conserved mechanisms across species. **a.** Volcano plots of abundance changes in proteomics of resistant strains compared to isogenic sensitive strains. Effect size and statistical significance (Methods) are represented on the x- and y- axis, respectively. Data points are colour-coded according to their conservation across or within-species. For sensitive strains see https://github.com/ElisabettaCacace/nitroxoline_2024/blob/main/figures/additional_figures.pdf. **b.** Protein abundance changes in nitroxoline-resistant strains. Only significant and strong changes (adjusted p-value ≤ 0.01 , $|\log_2$ fold-change (abundance)| ≥ 2) are included (Supplementary Data 6). Proteins and strains are clustered according to Pearson's correlation. Results are represented as in Fig. 5b. **c.** Amino acid changes from *emrR* mutations in clinical and experimentally evolved nitroxoline-resistant isolates. Results are represented as in Fig. 5d. **d.** Superimposition of mutated and wild-type OqxR structure. The G60-L67 duplication loop is highlighted in green in the structure (Methods) and in the OqxR amino acid sequence below. **e-f.** Amino acid changes from *adeL* (**e**) and *acrR/tetR* (**f**) mutations in clinical and experimentally evolved nitroxoline-resistant isolates. Results are represented as in Fig. 5d. **g.** Efflux-pump inhibitors resensitize nitroxoline-resistant strains. Results are represented as in Fig. 5e.

Resistant strains

conserved across
sensitive and resistant

conserved within
sensitive

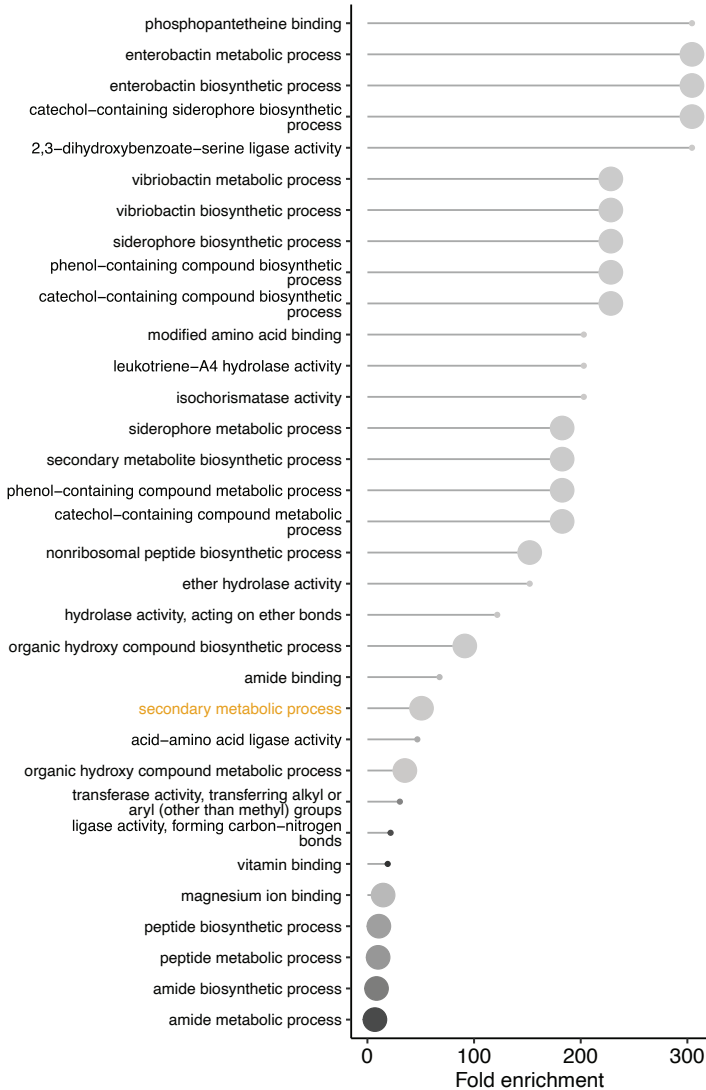


Supplementary Fig. 7. GO enrichment of significant hits from proteomics on nitroxoline-resistant strains (Fig. 5b, Supplementary Fig. 6b, Supplementary Data 6). Only sets yielding significant enrichments (down- or up-regulation in the indicated species) are shown (adjusted p-value < 0.05, one-sided Fisher's exact test). The number of protein hits is annotated for each term).

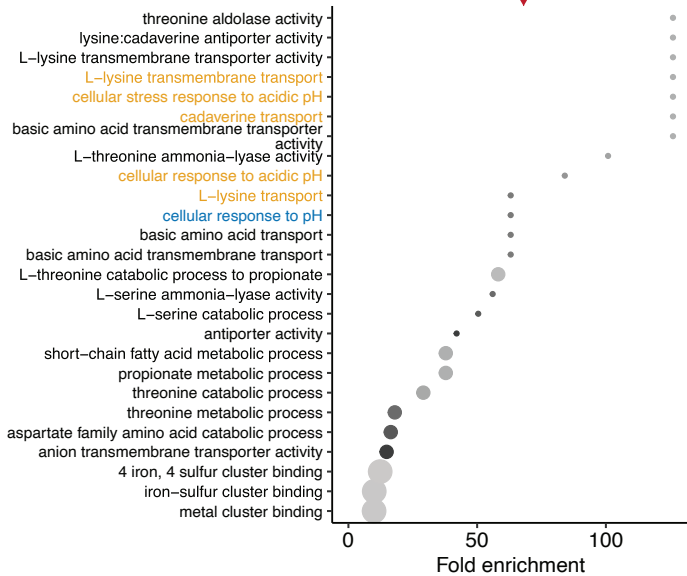
Sensitive strains

A. baumannii ↑

K. pneumoniae ↑



K. pneumoniae ↓



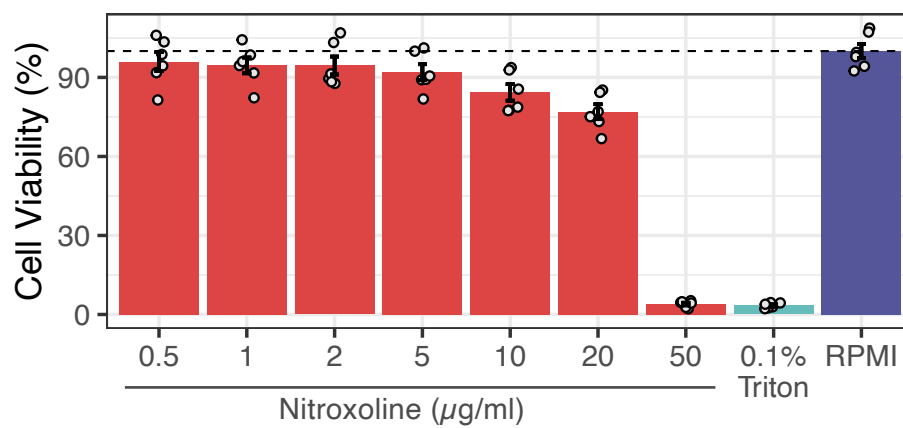
conserved across sensitive and resistant

conserved within sensitive

Number of proteins • 2 • 4 • 6 • 7

FDR 0.00 0.01

Supplementary Fig. 8. GO enrichment of significant hits from proteomics on nitroxoline-sensitive strains. Data is represented as in Supplementary Fig. 7.



Supplementary Fig. 9. MTT cell viability assay on HeLa cells exposed to increasing concentrations of nitroxoline. Controls include 0.1% Triton and RPMI as positive and negative controls, respectively. Results are expressed as mean and standard error across six biological replicates. 5 µg/ml is the nitroxoline dose used for the intracellular *S.Typhi* killing assay shown in Fig. 1d.