Supplementary Information

Antibiotic failure mediated by a resistant subpopulation in *Enterobacter cloacae*

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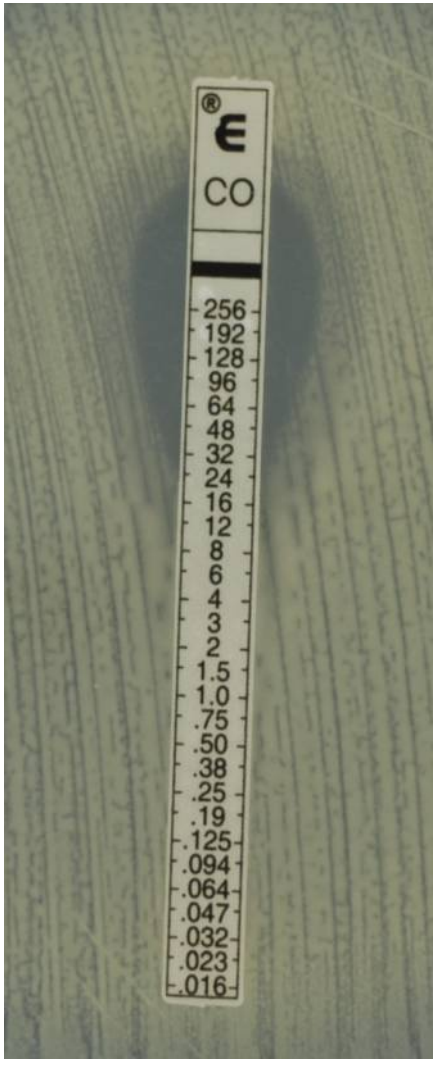
a

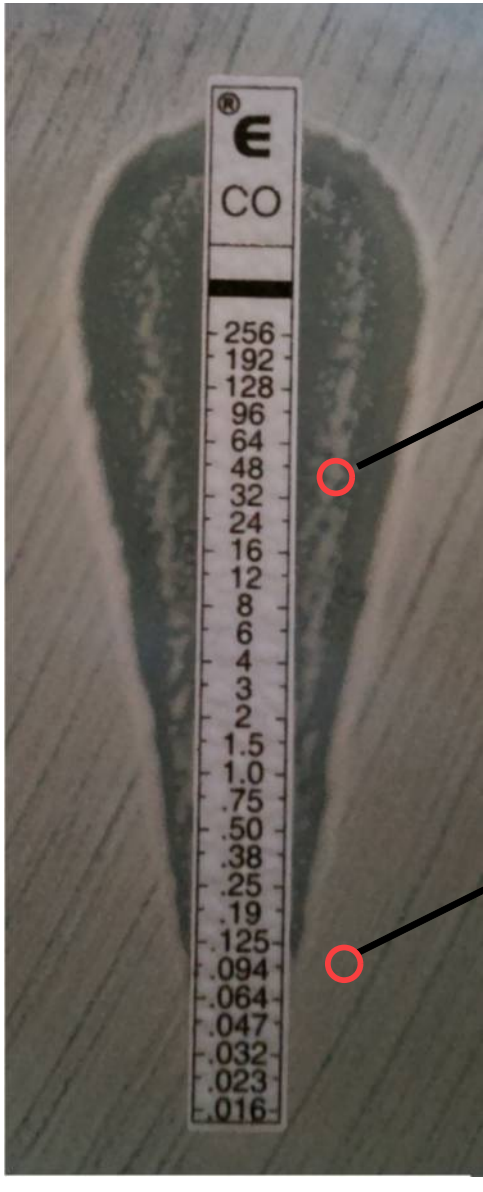
Susceptible

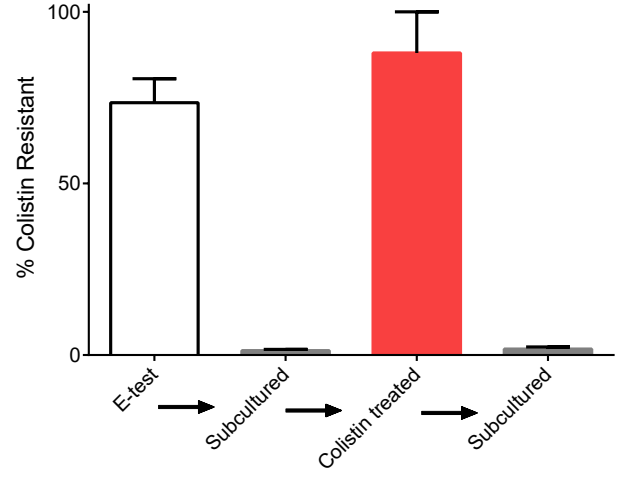
b

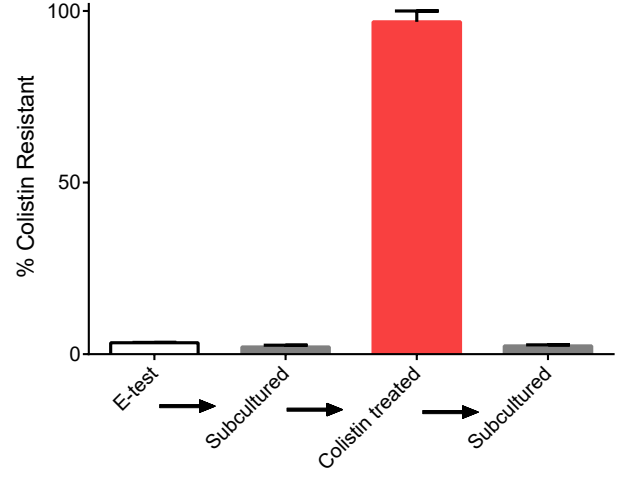


Resistant

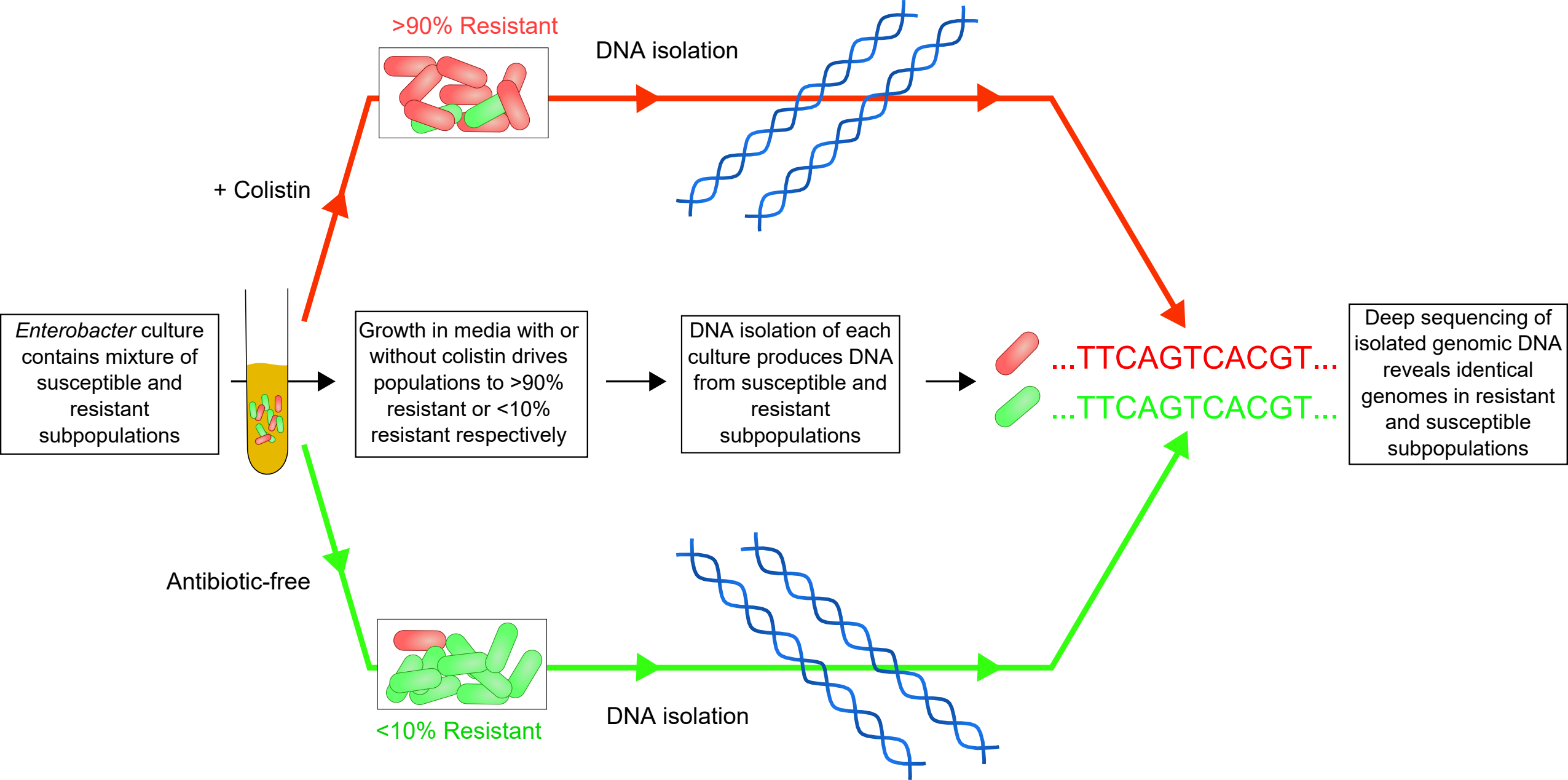
Supplementary Figure 1. Etests of colistin susceptible and resistant isolates. Colistin Etest analysis of (**a**) susceptible or (**b**) resistant *E. cloacae* clinical isolates, with drug concentration indicated in µg/mL. Data shown are representative of 3 Etests.


a
CO
256
192
128
96
64
48
32
24
16
12
8
6
4
3
2
1.5
1.0
.75
.50
.38
.25
.19
.125
.094
.064
.047
.032
.023
.016


b
% Colistin Resistant
100
50
0
E-test
Subcultured
Colistin treated
Subcultured

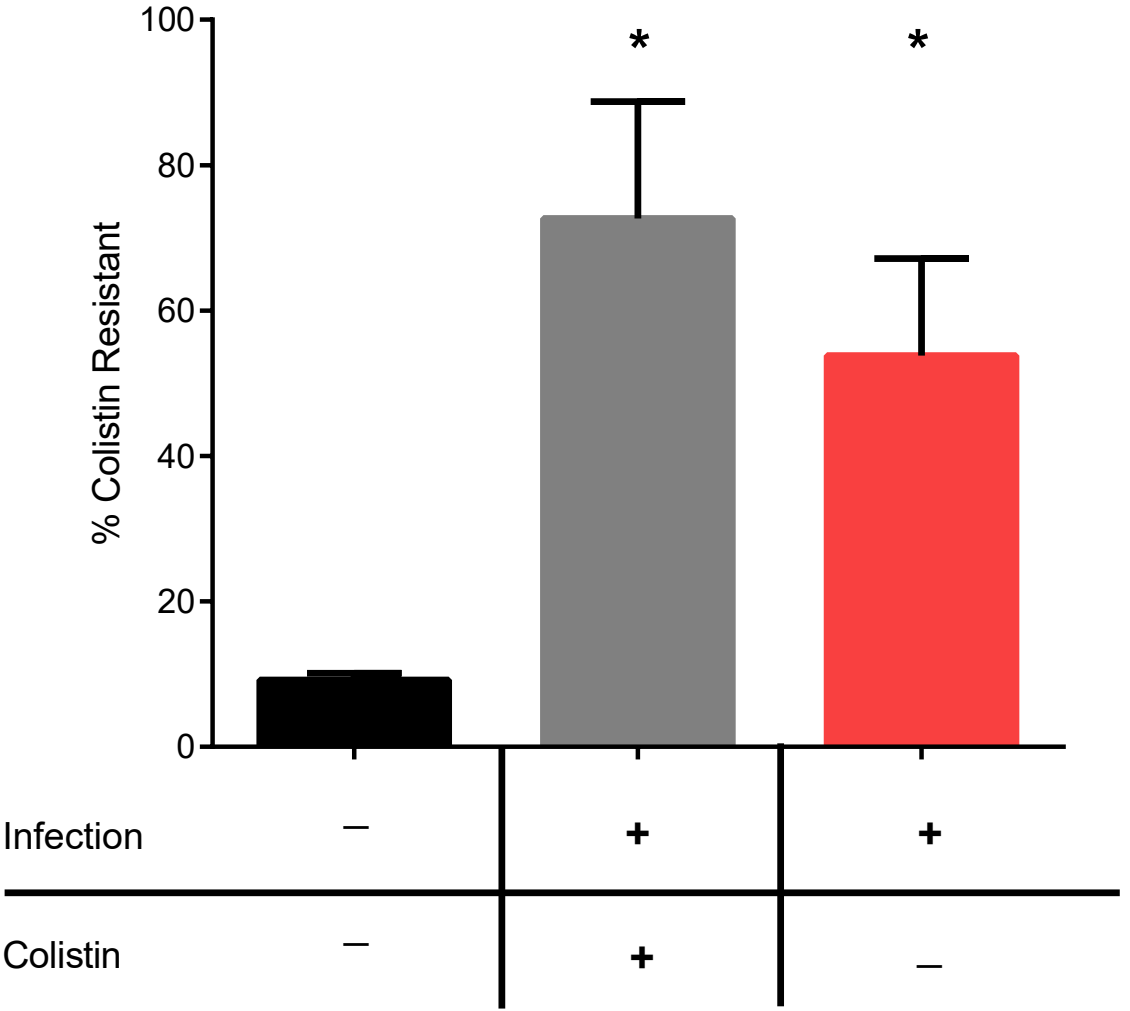

c
% Colistin Resistant
100
50
0
E-test
Subcultured
Colistin treated
Subcultured

Supplementary Figure 2. Bacteria from high and low antibiotic growth conditions behave identically after passage. **a,** R/S was plated on a colistin Etest plate and bacteria (circled in red) were harvested from within or outside the zone of clearing and assayed for colistin resistant subpopulations, (n=3). **b,c,** Bacteria taken from (**b**) within the zone of inhibition, representing the colistin resistant subpopulation and (**c**) outside the zone of inhibition, representing the colistin susceptible subpopulation were cultured. Bacteria were first cultured in drug free media, then subcultured in 100μg/mL colistin containing media, and then subcultured in drug free media again, with samples taken from each culture to assess colistin resistant subpopulations (n=3). Error bars represent s.e.m,.

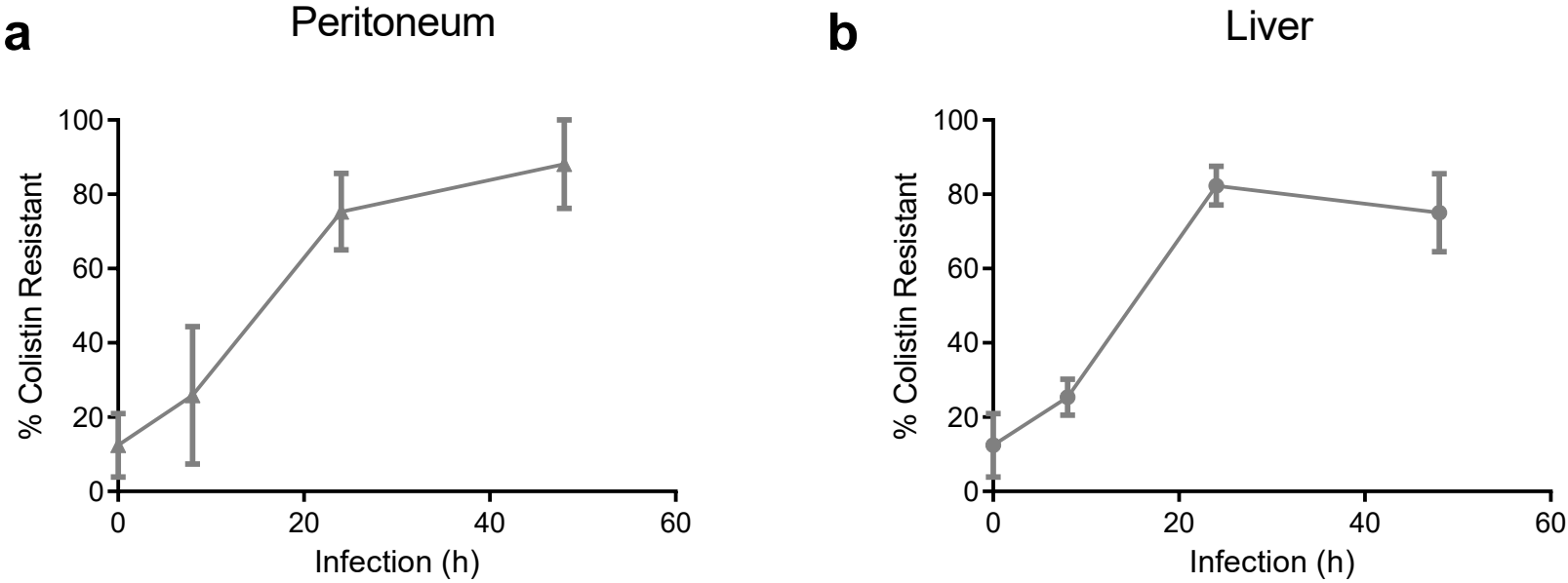

>90% Resistant
DNA isolation
+ Colistin
Enterobacter culture contains mixture of susceptible and resistant subpopulations
Growth in media with or without colistin drives populations to >90% resistant or <10% resistant respectively
DNA isolation of each culture produces DNA from susceptible and resistant subpopulations
...TTCAGTCACGT...
...TTCAGTCACGT...
Deep sequencing of isolated genomic DNA reveals identical genomes in resistant and susceptible subpopulations
Antibiotic-free
<10% Resistant
DNA isolation

Supplementary Figure 3. DNA sequencing of susceptible and resistant subpopulations.

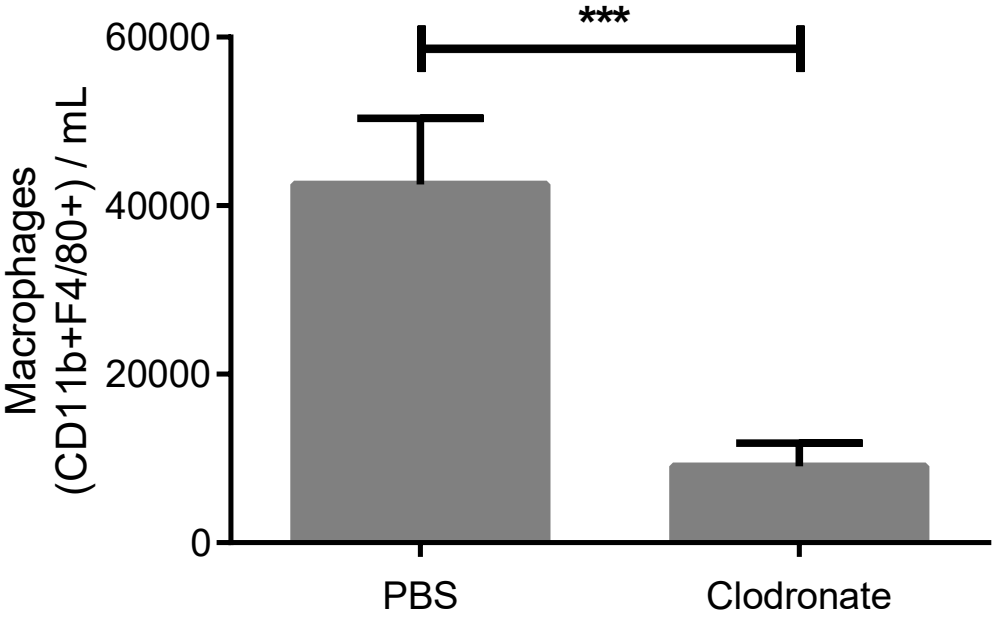
Flow chart of the procedure for DNA sequencing of the susceptible and resistant subpopulations of R/S. Cultures of R/S were grown in media with or without colistin to generate predominantly resistant or susceptible populations, respectively. If the DNA sequences of the two subpopulations were different, this would be detected as sequence differences when comparing the cultures in which either the susceptible or resistant subpopulation comprised the overwhelming majority of the sample. DNA was isolated from each culture and sequenced via DNAseq analysis. This analysis revealed identical genome sequences between each culture, indicating that the genome sequence of the susceptible and resistant subpopulations are identical. The same approach was used to harvest RNA for RNAseq analysis, which revealed significant transcriptome differences between the two subpopulations (see Tables S2 and S3).


100
80
60
40
20
0
% Colistin Resistant
*
*
Infection
–
+
+
Colistin
–
+
–

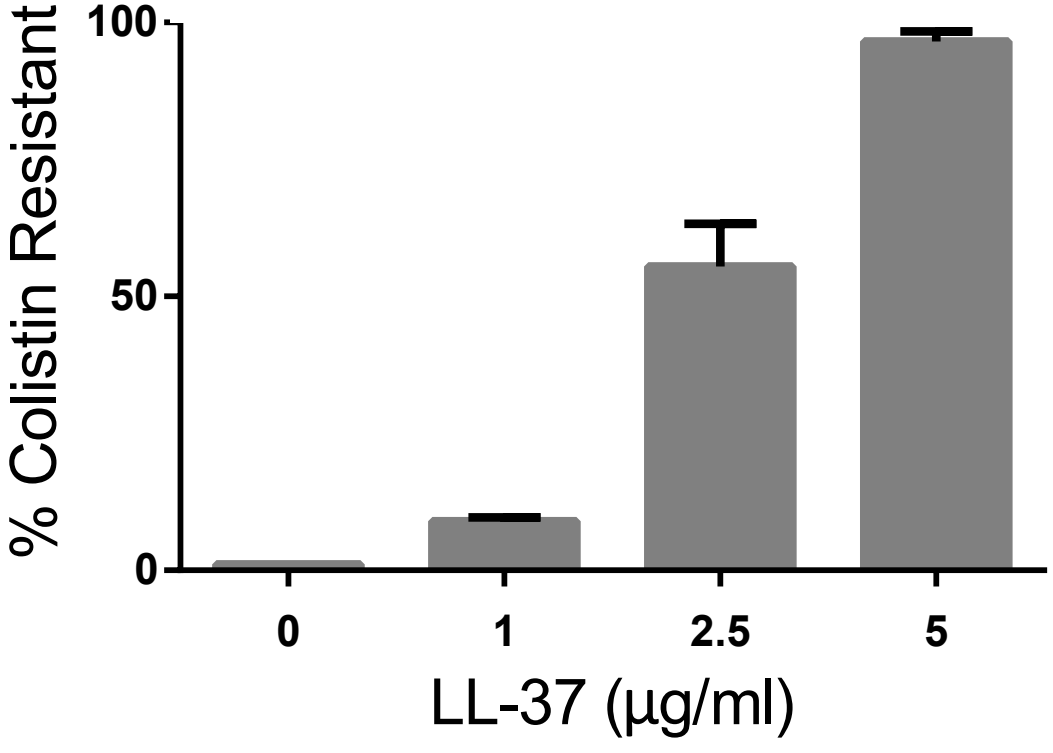
Supplementary Figure 4. Increase in the frequency of the colistin resistant subpopulation in the liver during *in vivo* infection. An inoculum of strain R/S (black bar) was used to infect mice intraperitoneally. Mice were treated with colistin (grey bar) or PBS (red bar) at 8, 14 and 20 hours. At 24 hours, liver samples were harvested and plated to quantify the number of colistin-resistant and total bacteria (n=5). Error bars represent s.e.m., (Mann-Whitney test, * $p < 0.05$).


a
Peritoneum
% Colistin Resistant
100
80
60
40
20
0
0
20
40
60
Infection (h)
b
Liver
% Colistin Resistant
100
80
60
40
20
0
0
20
40
60
Infection (h)

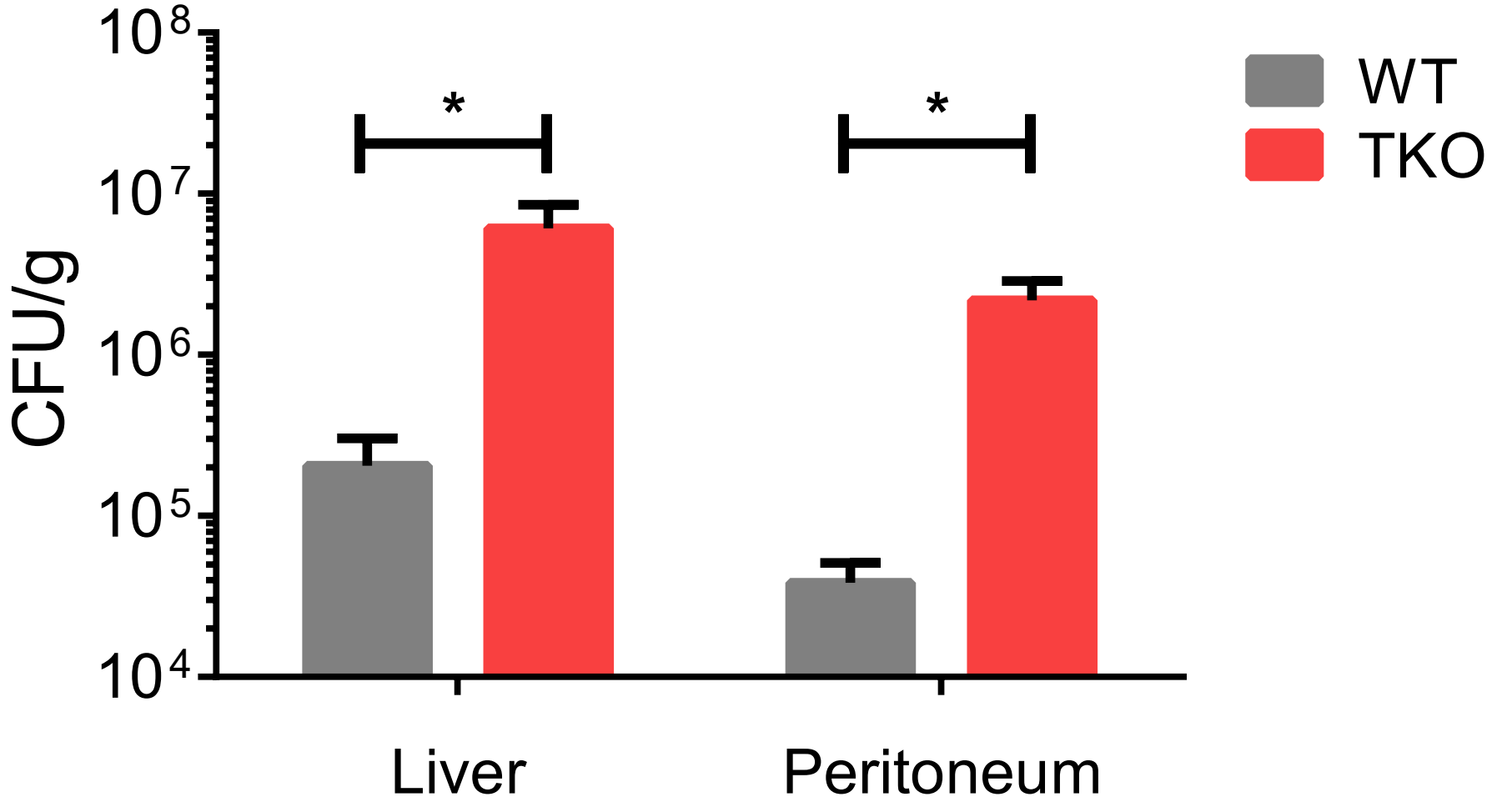
Supplementary Figure 5. Frequency of the colistin resistant subpopulation increases during *in vivo* infection. a,b % colistin resistance of R/S during a 48 hour mouse infection. Bacteria were recovered at each time point from (**a**) peritoneal lavage (n=5) or (**b**) liver samples (n=5). Error bars represent s.e.m,


***
60000
40000
20000
0
Macrophages
(CD11b+F4/80+) / mL
PBS
Clodronate

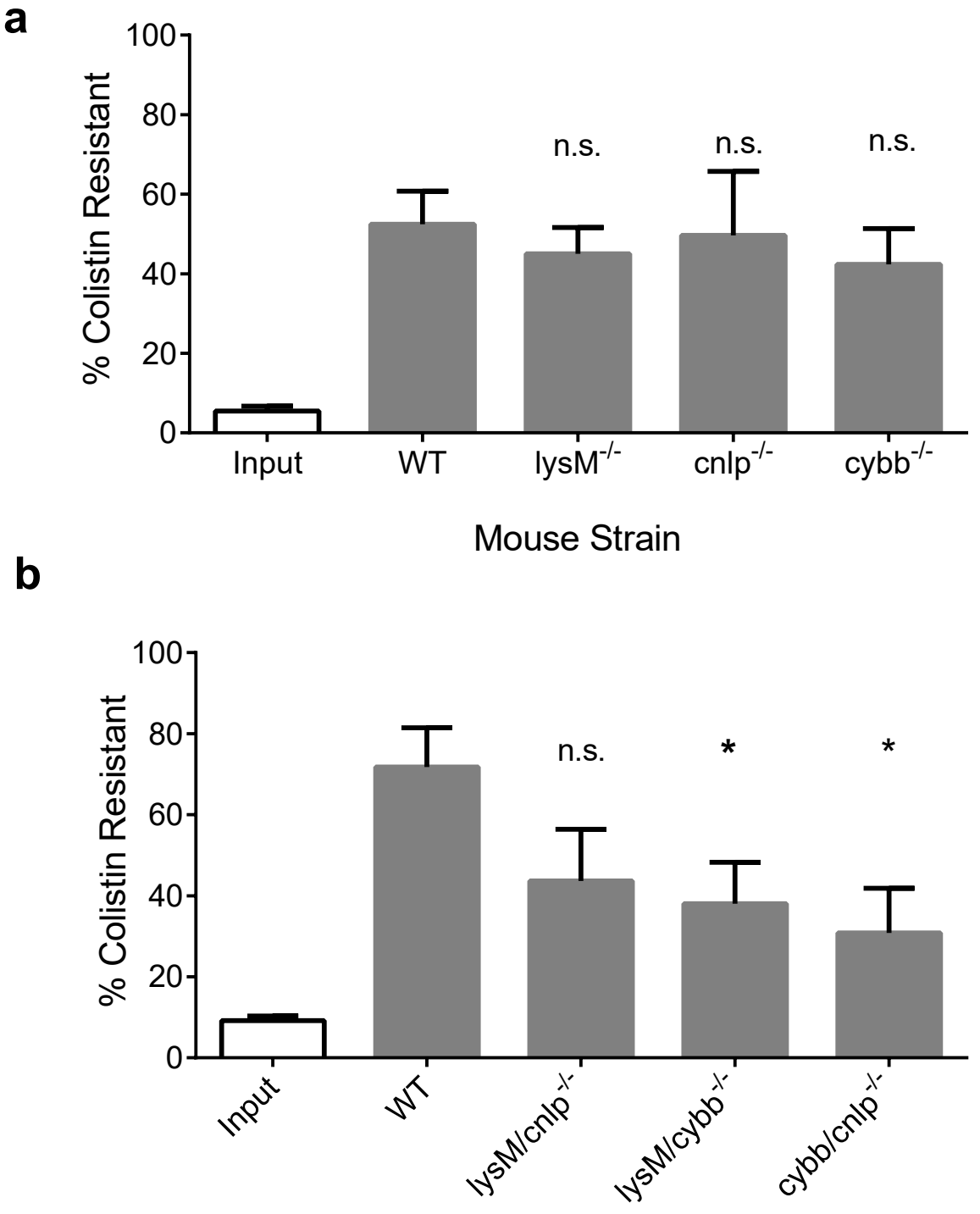
Supplementary Figure 6. Macrophage depletion via clodronate liposomes. Number of macrophages in peritoneal lavage fluid of PBS or clodronate liposome treated mice. Macrophages were defined as $CD11b^{+}F4/80^{+}$ cells by flow cytometry. Data compiled from 4 separate experiments (n=20). Error bars represent s.e.m. (Student's two-tailed t-test, ***, $p < 0.001$.)


% Colistin Resistant
100
50
0
0
1
2.5
5
LL-37 (µg/ml)

Supplementary Figure 7. The human antimicrobial peptide LL-37 leads to an increase in frequency of the colistin resistant subpopulation. Strain R/S was treated with the indicated amounts of human LL-37 for 5 hours. Samples were plated to quantify the numbers of total and colistin-resistant bacteria and % colistin resistance was calculated (n = 3). Error bars represent s.e.m.

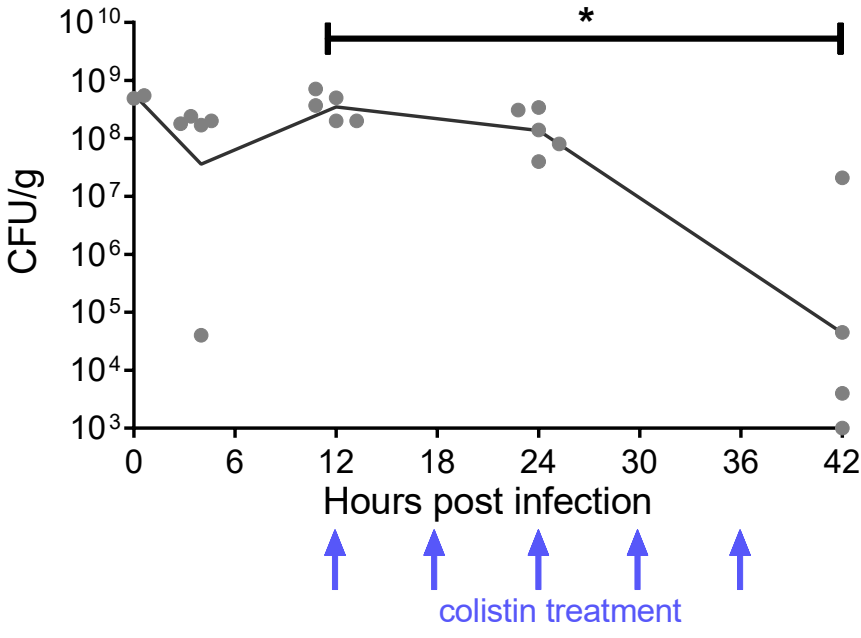

10^8
10^7
10^6
10^5
10^4
CFU/g
*
*
WT
TKO
Liver
Peritoneum

Supplementary Figure 8. Triple knockout mice lacking antimicrobials exhibit increased bacterial levels during infection. Wild type or triple knockout (TKO) mice lacking lysozyme (*lysM*), CRAMP (*cnlp*) and the gp91 component of the NADPH oxidase (*cybb*) were infected with R/S, and CFU in the liver and peritoneal lavage fluid were quantified at 8 hours post infection (n = 5). Error bars represent s.e.m. (Mann-Whitney test, * $p < 0.05$).

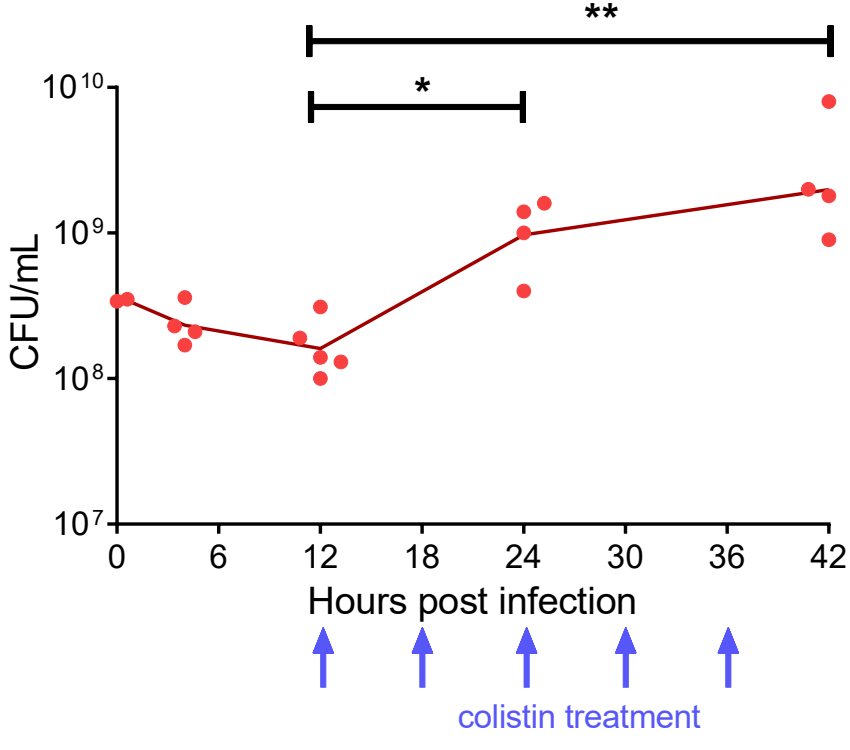

a
100
80
60
40
20
0
% Colistin Resistant
n.s.
n.s.
n.s.
Input
WT
lysM-/-
cnlp-/-
cybb-/-
Mouse Strain
b
100
80
60
40
20
0
% Colistin Resistant
n.s.
*
*
Input
WT
lysM/cnlp-/-
lysM/cybb-/-
cybb/cnlp-/-

Supplementary Figure 9. Combinations of host antimicrobials control the increase in frequency of the R/S colistin resistant subpopulation. Single knockout mice lacking lysozyme (*lysM*), CRAMP (*cnlp*) or the NADPH oxidase (*cybb*) (**a**) and double knockout mice lacking the indicated combinations of the antimicrobials (**b**) were infected with R/S for 8 hours, and the % colistin resistance was compared to that of the initial inoculum (n = 4 or 5). Error bars represent s.e.m. (Mann-Whitney test, * $p < 0.05$., n.s. = not significant).

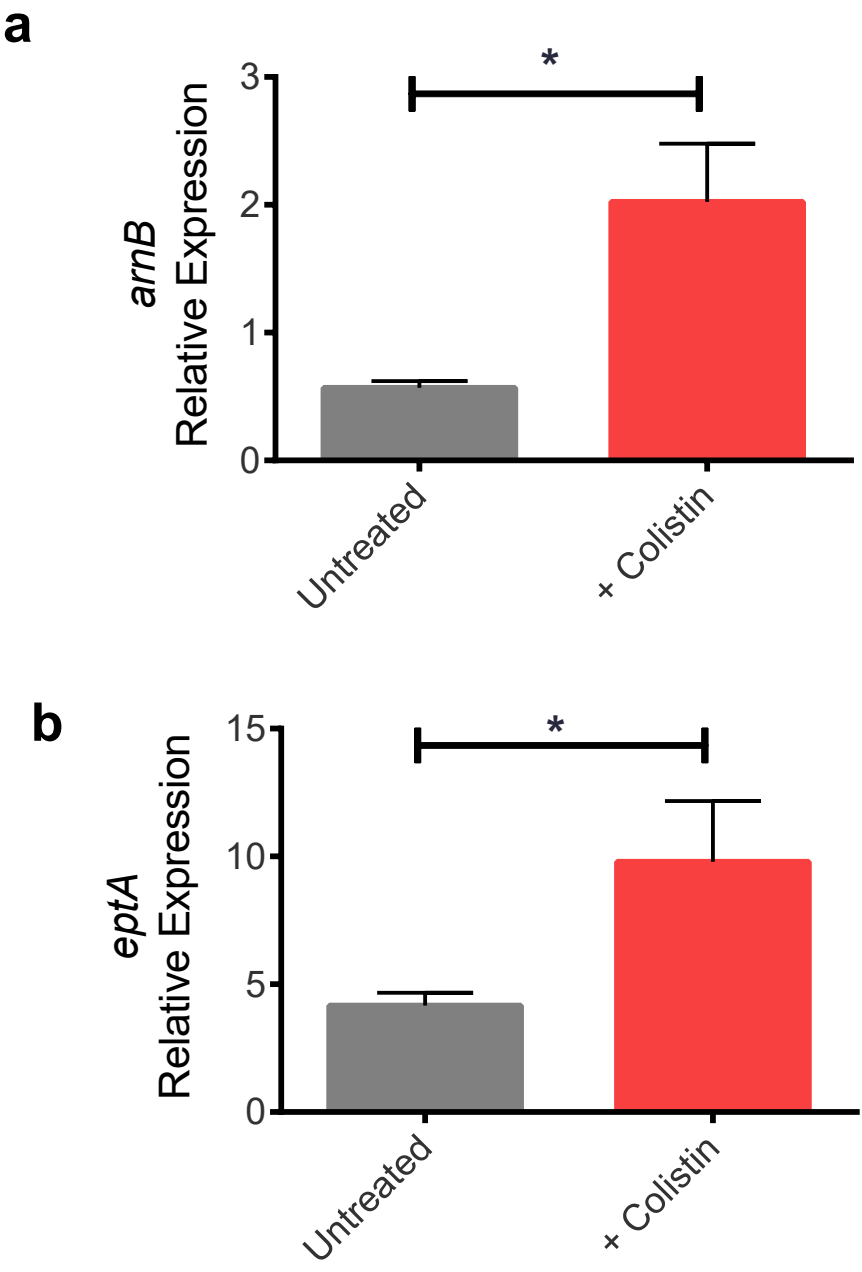
a


*
CFU/g
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
0
6
12
18
24
30
36
42
Hours post infection
colistin treatment

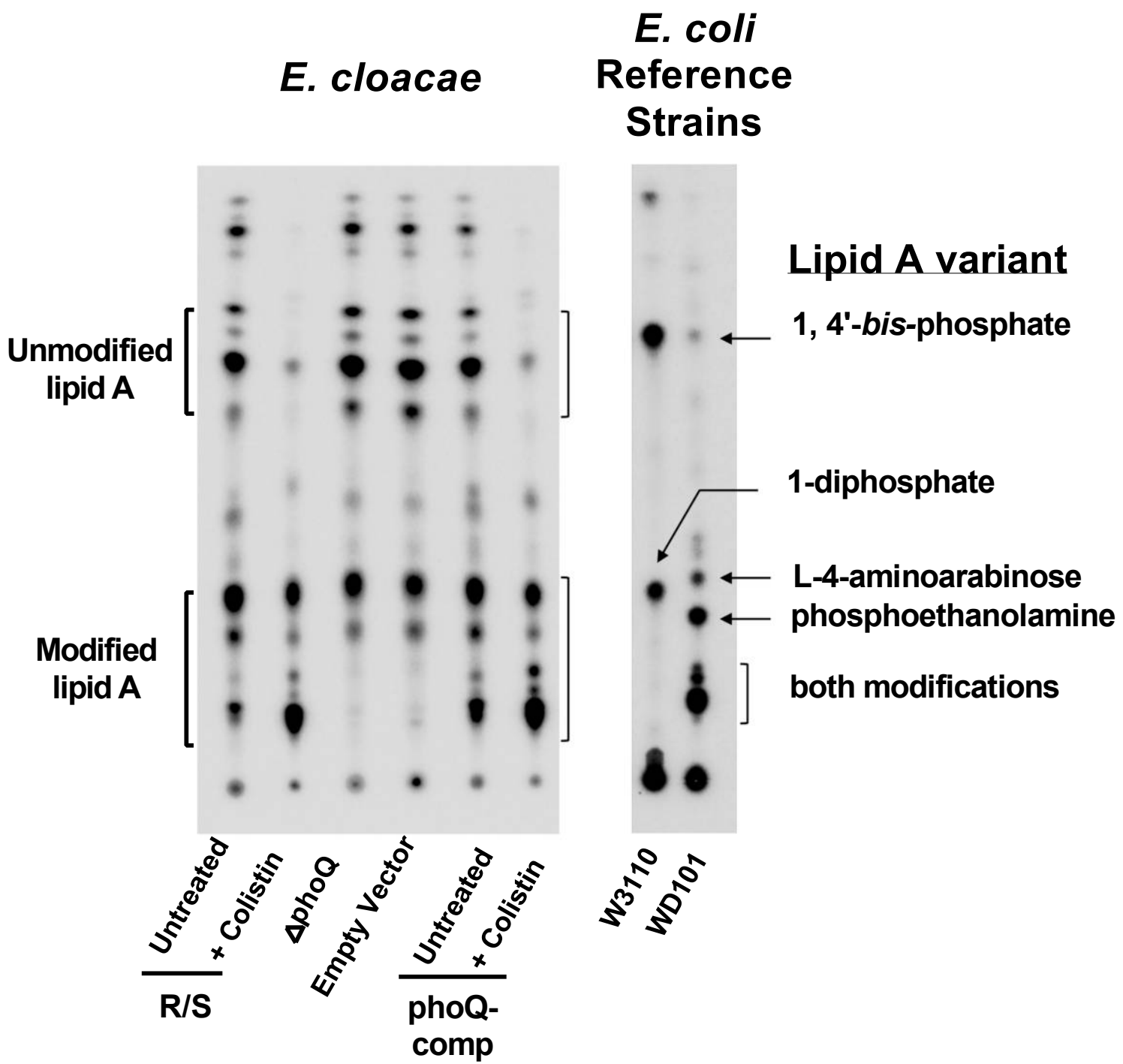
b


**
*
CFU/mL
10^{10}
10^{9}
10^{8}
10^{7}
0
6
12
18
24
30
36
42
Hours post infection
colistin treatment

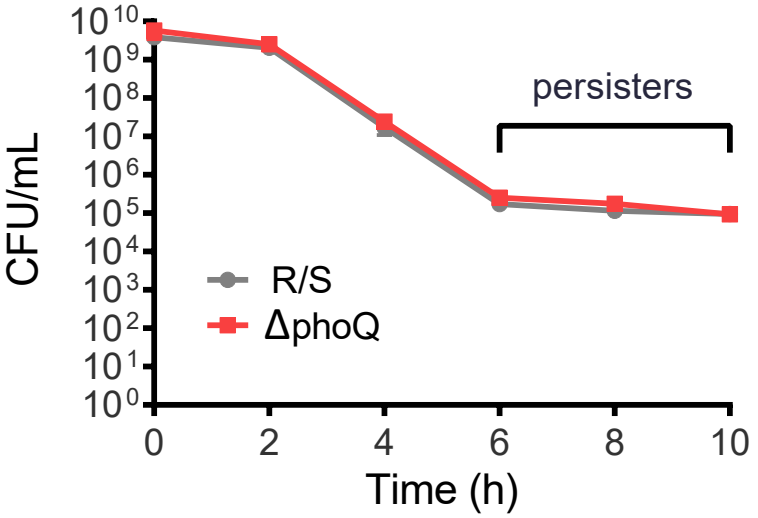
Supplementary Figure 10. *In vivo* growth and expansion of R/S during colistin treatment of mice. Wild-type mice were infected with a lethal dose of (**a)** a susceptible strain (n = 4 to 6) or (**b)** R/S (n = 4 to 6) and then given doses of colistin every six hours starting at 12 hours post infection. Mice were sacrificed to determine peritoneal CFU at 0, 6, 24 and 42 hours. Error bars represent s.e.m., lines represent median. (Mann-Whitney test, * $p < 0.05$, ** $p < 0.01$).


a
arnB
Relative Expression
3
2
1
0
*
Untreated
+ Colistin
b
eptA
Relative Expression
15
10
5
0
*
Untreated
+ Colistin

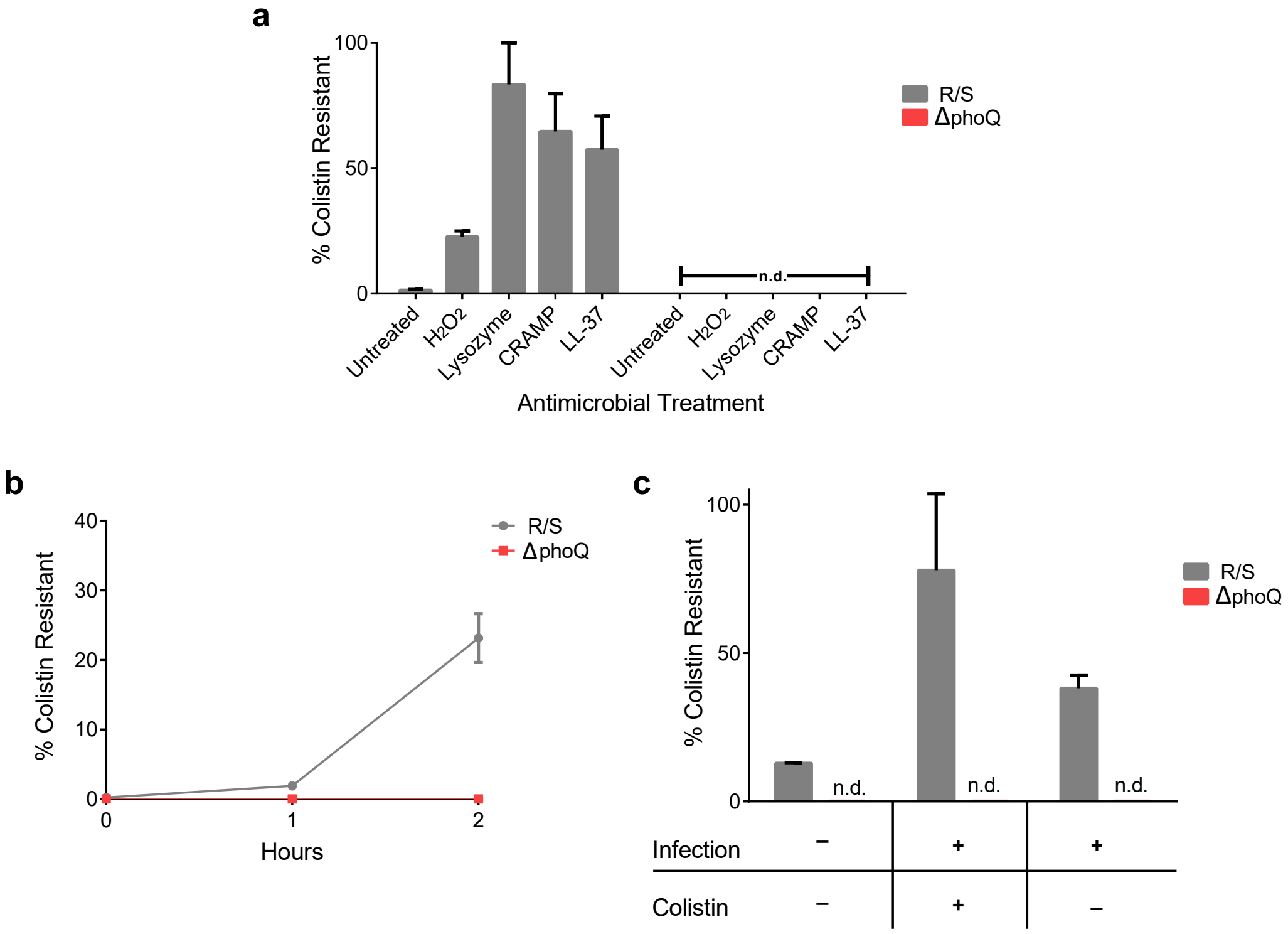
Supplementary Figure 11. Colistin resistant and susceptible subpopulations express different levels of lipid A modification genes. RNA was harvested from strain R/S cultured without (Untreated) or with colistin (+Colistin) as in Supplementary Figure 3 to generate cultures with increased prevalence of the colistin susceptible or resistant subpopulation, respectively. Relative expression compared to the housekeeping gene *rpoD* of (**a**) *arnB* (n = 3) and (**b**) *eptA* (n = 6) lipid A modification genes was quantified by qRT-PCR. (Error bars represent s.e.m. Mann-Whitney test, * $p < 0.05$).


E. cloacae
E. coli Reference Strains
Lipid A variant
1, 4'-bis-phosphate
1-diphosphate
L-4-aminoarabinose
phosphoethanolamine
both modifications
Unmodified lipid A
Modified lipid A
Untreated
+ Colistin
ΔphoQ
Empty Vector
Untreated
+ Colistin
W3110
WD101
R/S
phoQ-comp

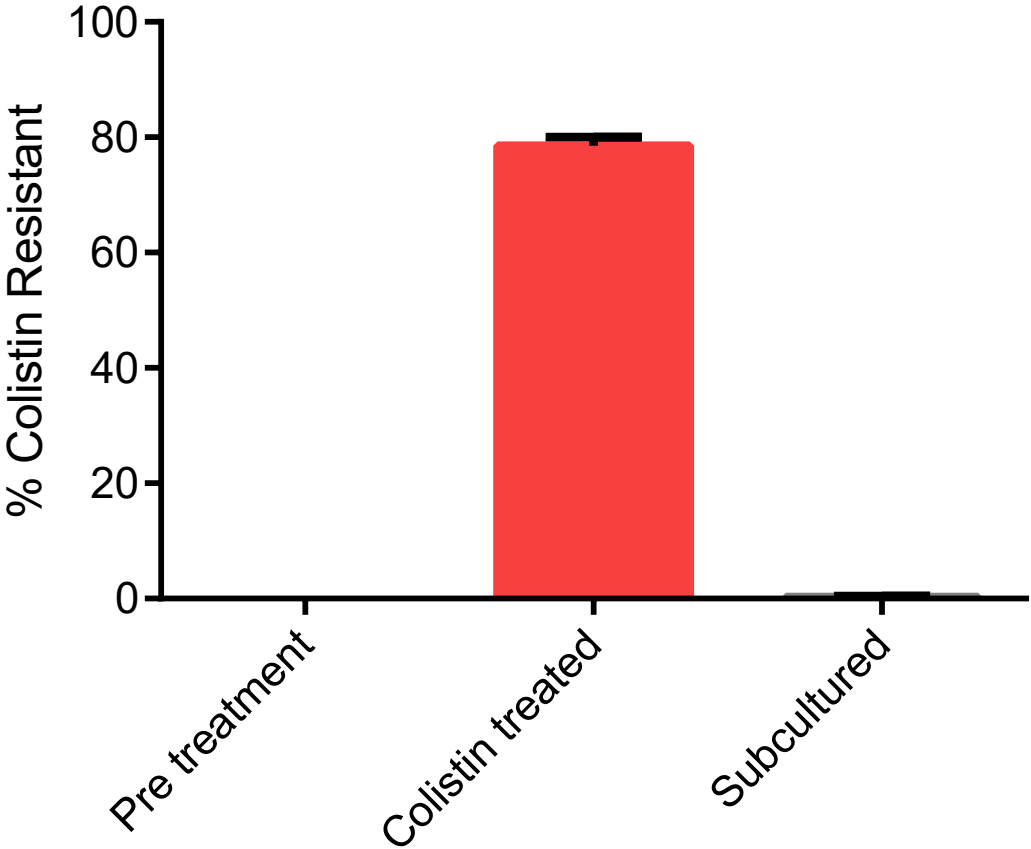
Supplementary Figure 12. Lipid A analysis reveals modifications present in the R/S resistant subpopulation. Lipid A species were harvested from strains cultured without or with colistin treatment as in Supplementary Figure 3 to generate cultures with increased prevalence of the colistin susceptible or resistant subpopulation, respectively. **a**, Thin layer chromatography separation of lipid A species was performed on R/S cultured without (Untreated) or with (+Colistin) colistin pretreatment, the *phoQ* deletion mutant (Δ*phoQ*), Δ*phoQ* complemented with an empty vector (Empty Vector), complemented with *phoQ* (Untreated) or complemented with *phoQ* and then treated with colistin (+Colistin). **b**, Thin layer chromatography of reference *E. coli* strains W3110 (wild-type parent strain with unmodified lipid A) and WD101 (modified lipid A) with known lipid A modifications were used as controls[50]. Data is representative of multiple experiments (n = 3).


CFU/mL
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
persisters
R/S
ΔphoQ
0
2
4
6
8
10
Time (h)

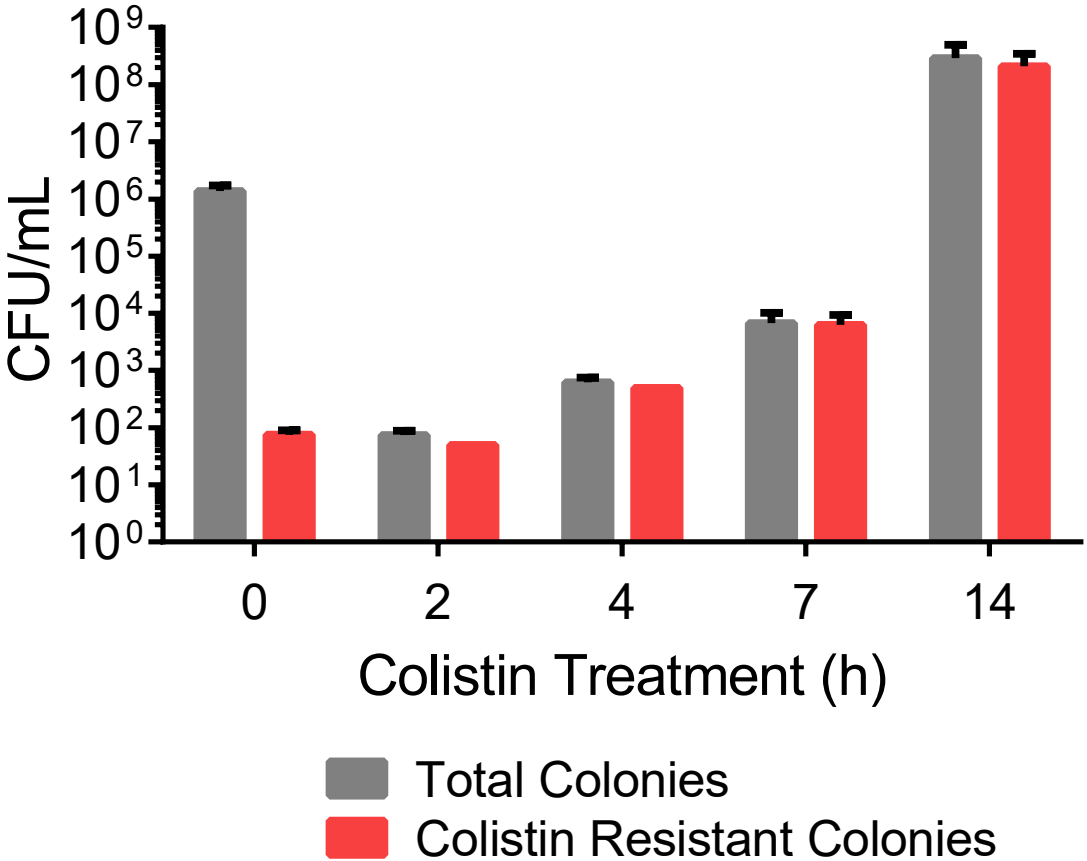
Supplementary Figure 13. Kanamycin persisters in R/S are not dependent on PhoQ. R/S and Δ*phoQ* were treated with 900 μg/mL kanamycin and CFU/mL enumerated (n = 3). The period between 6 and 8 hours with a plateau in killing represents the population of surviving persisters.


a
% Colistin Resistant
100
50
0
R/S
ΔphoQ
n.d.
Untreated
H2O2
Lysozyme
CRAMP
LL-37
Untreated
H2O2
Lysozyme
CRAMP
LL-37
Antimicrobial Treatment
b
% Colistin Resistant
40
30
20
10
0
R/S
ΔphoQ
0
1
2
Hours
c
% Colistin Resistant
100
50
0
R/S
ΔphoQ
n.d.
n.d.
n.d.
Infection
–
+
+
Colistin
–
+
–

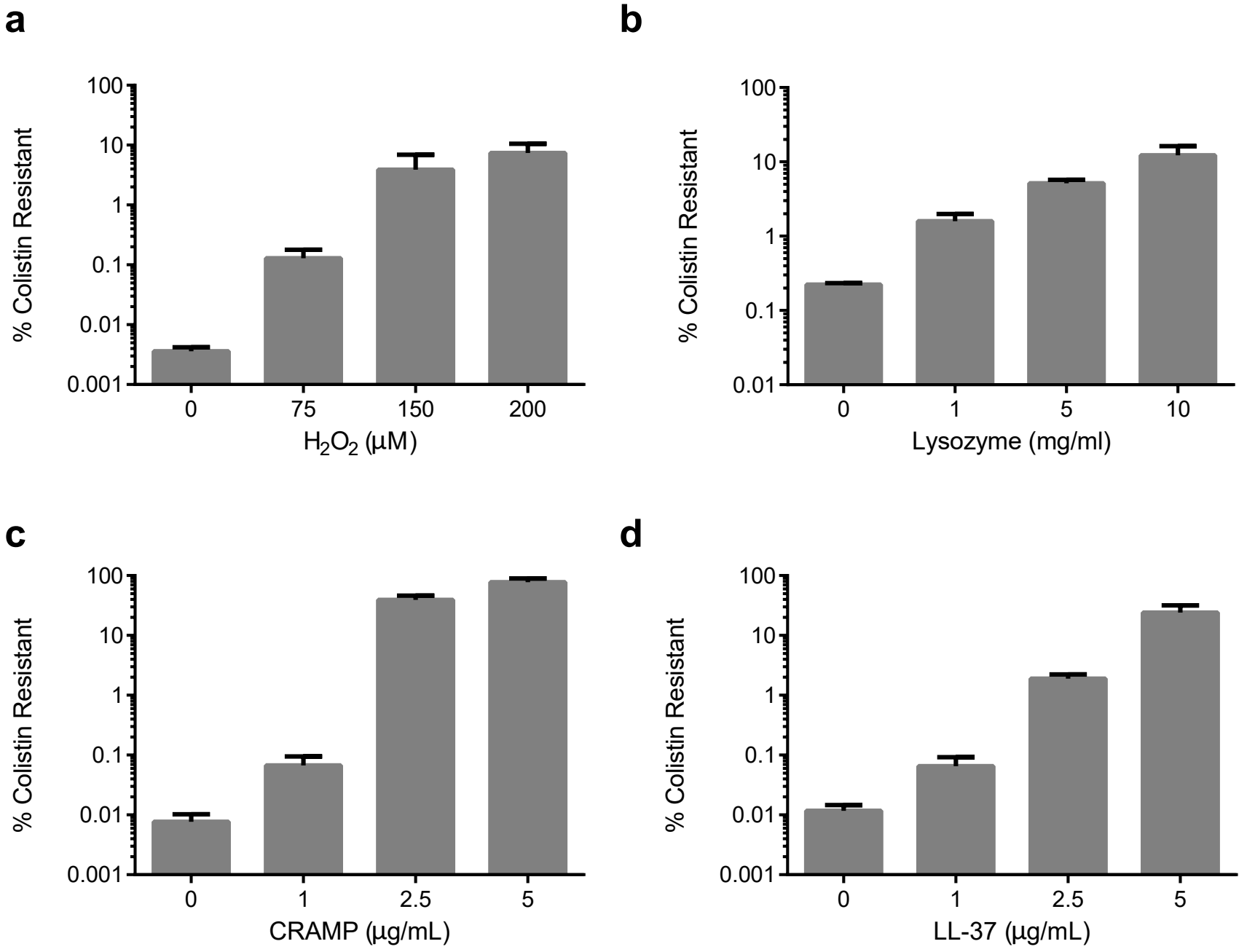
Supplementary Figure 14. PhoQ is required for the R/S colistin resistant subpopulation. a, % colistin resistance of R/S and Δ*phoQ* after 5 hour treatment with 100uM H_2O_2, 5 mg/mL lysozyme, 5 µg/mL CRAMP or 10 ug/mL LL-37 (n = 3). **b,** % colistin resistance of R/S and Δ*phoQ* during macrophage infection at the indicated timepoints (n = 3). **c,** % colistin resistance of R/S and Δ*phoQ* after 24 hour mouse infection (n = 5). No resistant colonies were detected (n.d.) for all Δ*phoQ* samples. Error bars represent s.e.m.


100
80
60
40
20
0
% Colistin Resistant
Pre treatment
Colistin treated
Subcultured

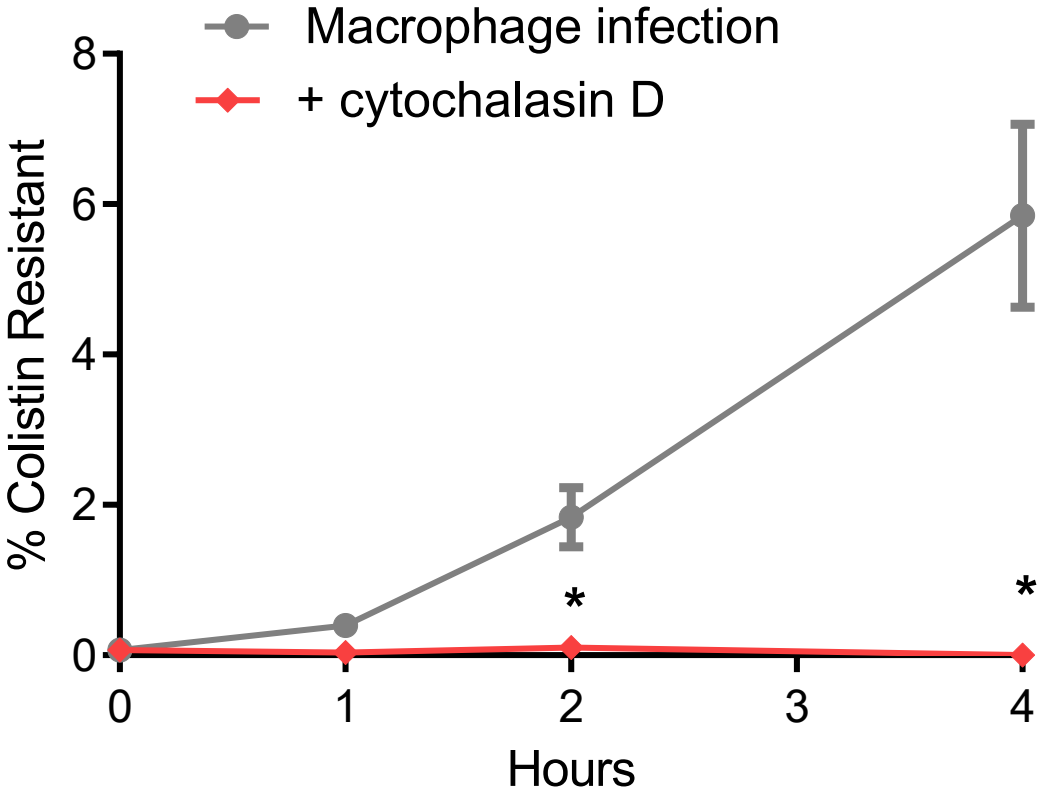
Supplementary Figure 15. The frequency of the colistin resistant subpopulation of R/S-lo increases in the presence of drug. % colistin resistant bacteria was calculated for R/S-lo before colistin treatment, after 20 h in 100 μg/mL colistin, and after 8 h drug free subculture (n = 3). Error bars represent s.e.m.


CFU/mL
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
2
4
7
14
Colistin Treatment (h)
Total Colonies
Colistin Resistant Colonies

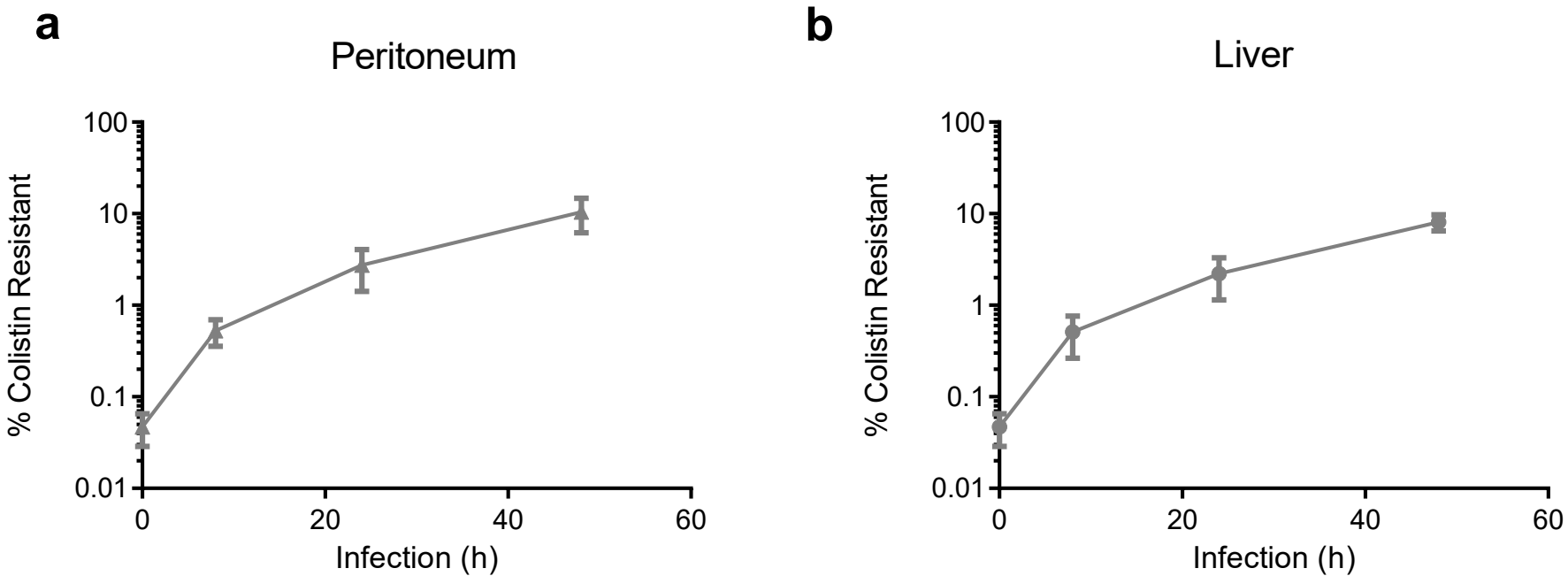
Supplementary Figure 16. Colistin selects for the colistin resistant subpopulation of R/S-lo. Colistin resistant and total CFU of R/S-lo during 14 h treatment with 100μg/mL colistin in liquid culture (n = 3). Error bars represent s.e.m.


a
% Colistin Resistant
100
10
1
0.1
0.01
0.001
0
75
150
200
H2O2 (µM)
b
% Colistin Resistant
100
10
1
0.1
0.01
0
1
5
10
Lysozyme (mg/ml)
c
% Colistin Resistant
100
10
1
0.1
0.01
0.001
0
1
2.5
5
CRAMP (µg/mL)
d
% Colistin Resistant
100
10
1
0.1
0.01
0.001
0
1
2.5
5
LL-37 (µg/mL)

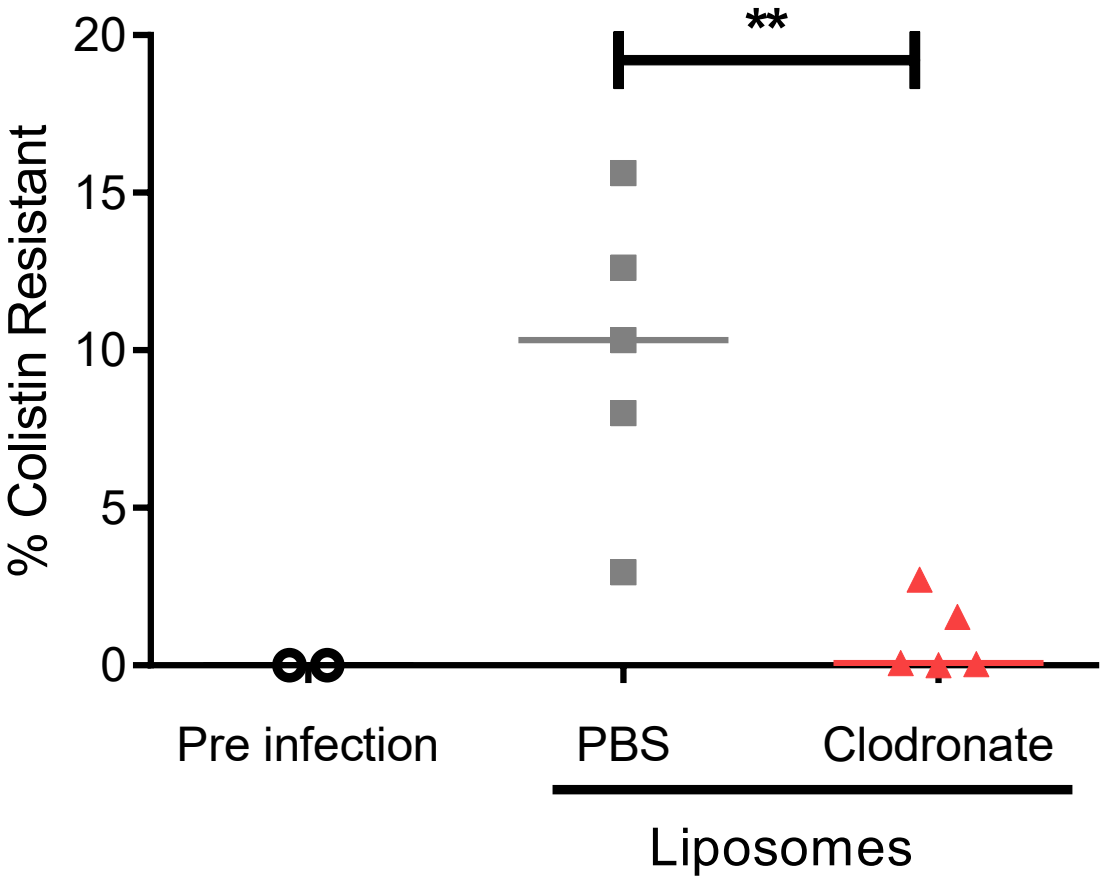
Supplementary Figure 17. Host antimicrobials lead to an increase in the frequency of the colistin resistant subpopulation of R/S-lo. R/S-lo was treated for 5 h with the indicated concentrations of (**a**) H_2O_2 (n = 3), (**b**) lysozyme (n = 3), (**c**) CRAMP (n = 3) or (**d)** LL-37 (n = 3) and % colistin resistance was calculated. Error bars represent s.e.m,.


Macrophage infection
+ cytochalasin D
% Colistin Resistant
8
6
4
2
0
0
1
2
3
4
Hours
*
*

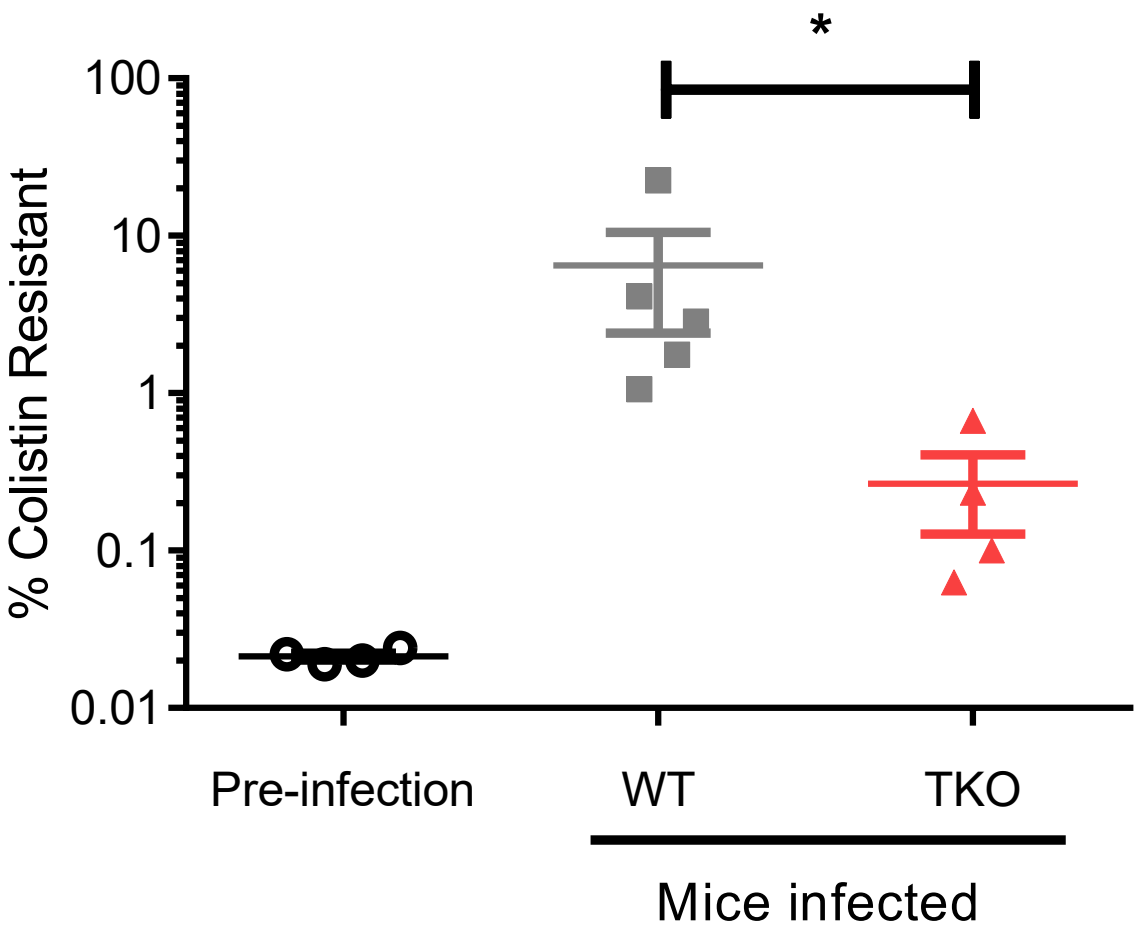
Supplementary Figure 18. The frequency of the R/S-lo colistin resistant subpopulation increases in macrophages. Bone marrow-derived macrophages were infected with R/S-lo. % colistin resistance of R/S-lo within macrophages pretreated or untreated with cytochalasin D is shown at each timepoint (n = 6). Error bars represent s.e.m. (Student's two-tailed t-test, * $p<0.05$).


a
Peritoneum
% Colistin Resistant
100
10
1
0.1
0.01
0
20
40
60
Infection (h)
b
Liver
% Colistin Resistant
100
10
1
0.1
0.01
0
20
40
60
Infection (h)

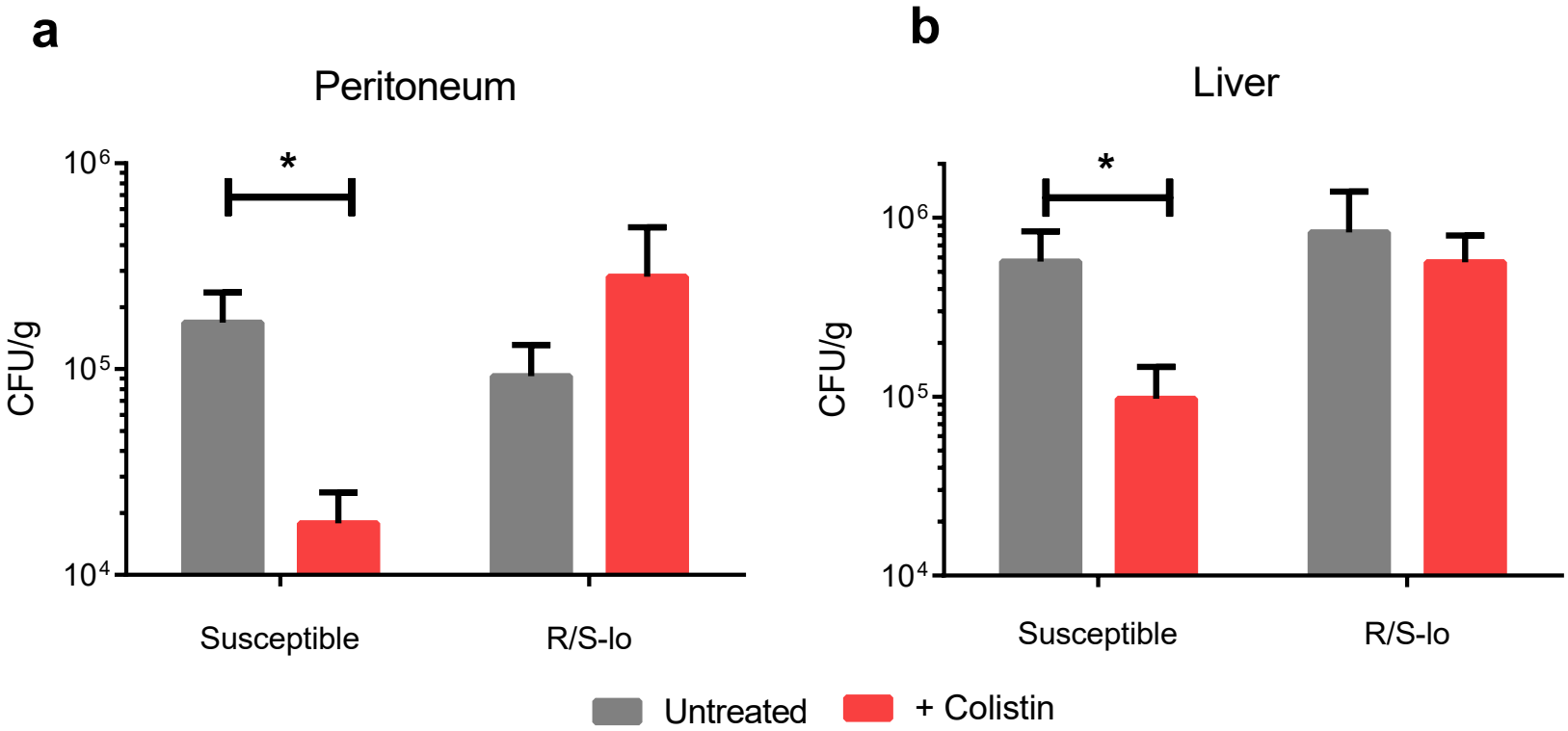
Supplementary Figure 19. The frequency of the R/S-lo resistant subpopulation increases during mouse infection. a,b % colistin resistance of R/S-lo during a 48 hour mouse infection. Bacteria were recovered at each time point from (**a**) peritoneal lavage (n = 5) or (**b**) liver samples (n = 5). Error bars represent s.e.m.


20
15
10
5
0
% Colistin Resistant
**
Pre infection
PBS
Clodronate
Liposomes

Supplementary Figure 20. Macrophages are required for the increase in the frequency of the R/S-lo resistant subpopulation during infection. Mice pre-treated with PBS (as a control) or clodronate containing liposomes (to deplete macrophages) were infected with R/S-lo. % colistin resistance of R/S-lo recovered in peritoneal lavage fluid after 8 hour infection is shown (n = 5). Error bars represent s.e.m., center value represents median. (Mann-Whitney test, ** $p < 0.01$).

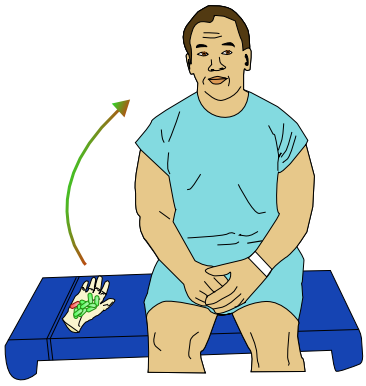

*
100
10
1
0.1
0.01
% Colistin Resistant
Pre-infection
WT
TKO
Mice infected

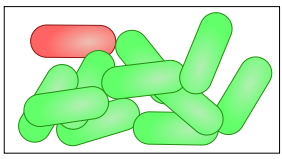
Supplementary Figure 21. Specific host antimicrobials contribute to the increased frequency of the R/S-lo subpopulation *in vivo*. Triple knockout mice (TKO) lacking the NADPH oxidase gp91 subunit (which contributes to superoxide production), lysozyme and CRAMP were infected with R/S-lo. % colistin resistance of R/S-lo recovered in peritoneal lavage fluid after 8 hour infection is shown (n = 5). Error bars represent s.e.m., center value represents median. (Mann-Whitney test, * $p < 0.05$).


a
Peritoneum
CFU/g
10^6
10^5
10^4
*
Susceptible
R/S-lo
b
Liver
CFU/g
10^6
10^5
10^4
*
Susceptible
R/S-lo
Untreated
+ Colistin

Supplementary Figure 22. Inefficacy of colistin in reducing the levels of strain R/S-lo during *in vivo* infection. Mice infected with R/S-lo or a susceptible clinical isolate were treated with colistin at 8, 14 and 20 hours. CFU were quantified at 24 hours in the (**a**) peritoneal lavage fluid (n = 5) and (**b**) liver (n = 5). Error bars represent s.e.m. Mann-Whitney test, * $p < 0.05$.

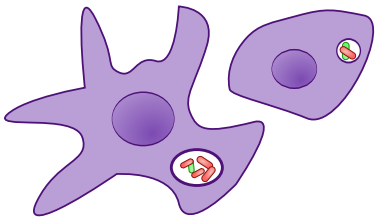
Patient infected with bacterial pathogen containing minor antibiotic resistant sub-population.

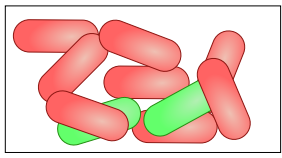




Susceptible

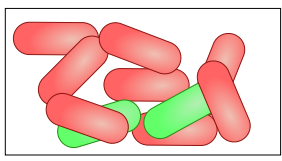
In vivo, the bacteria are subjected to immune pressure from macrophages and host antimicrobials. This leads to an increase in the frequency of the resistant subpopulation.





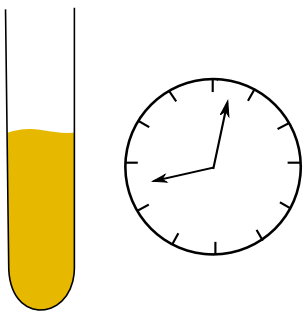
Resistant

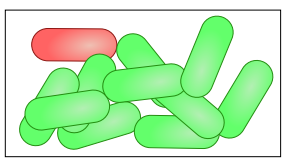
After the infection is detected, a sample is taken from the patient for analysis. A large antibiotic resistant population is present in this sample due to host immune pressure.



Resistant

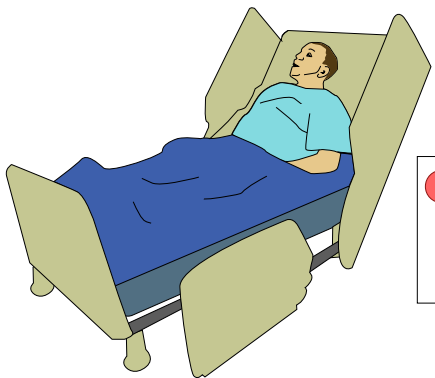
The sample is subcultured in media without antibiotic. The antibiotic resistant subpopulation decreases to the baseline level, preventing its detection by diagnostic testing, and leading to classification of the isolate as susceptible.

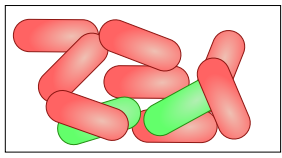




Susceptible

The patient is treated with antibiotic, under the assumption that the strain is susceptible. However, the bacteria in the patient are largely resistant. Antibiotic therapy proves ineffective and the infection worsens.



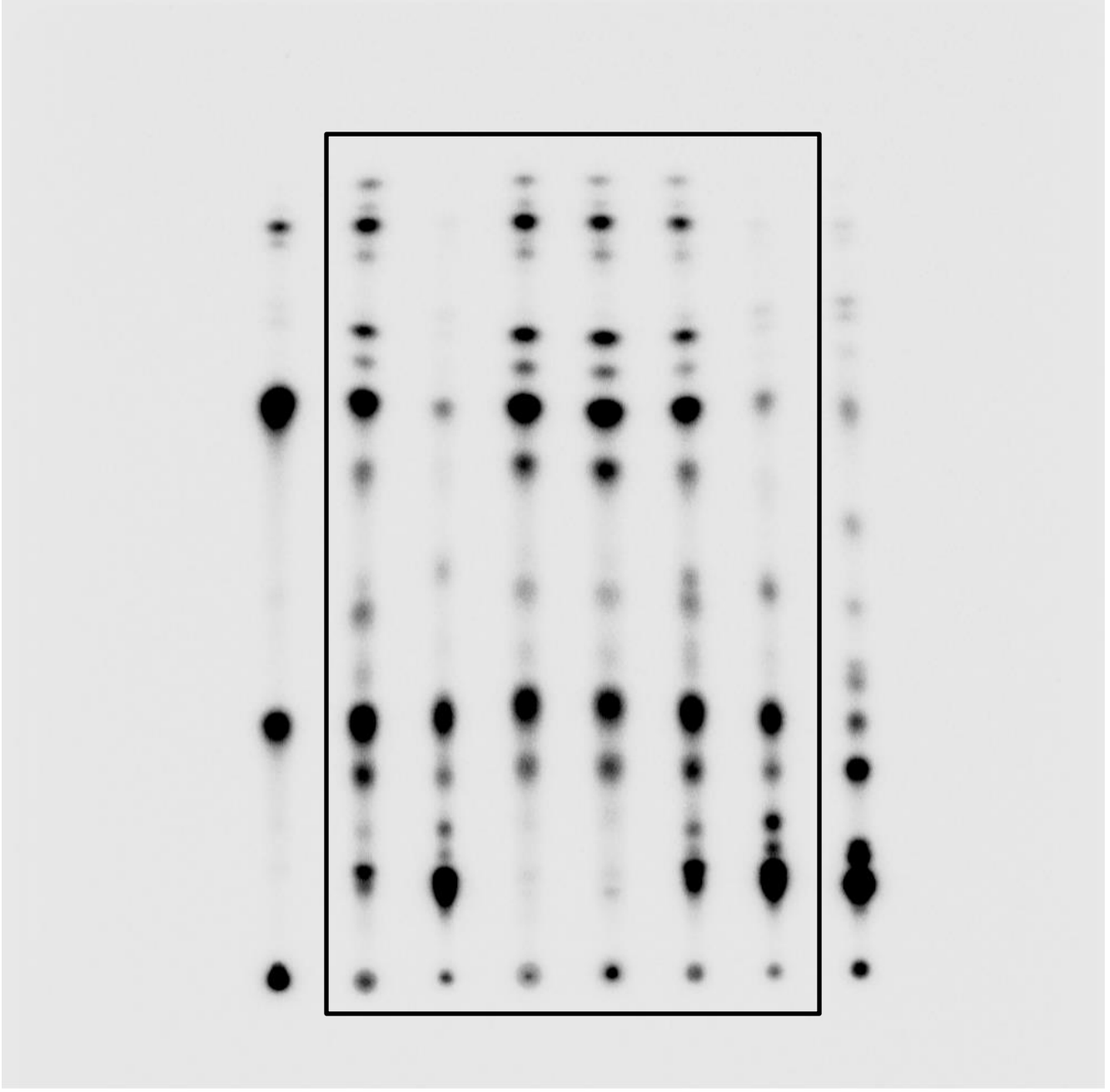


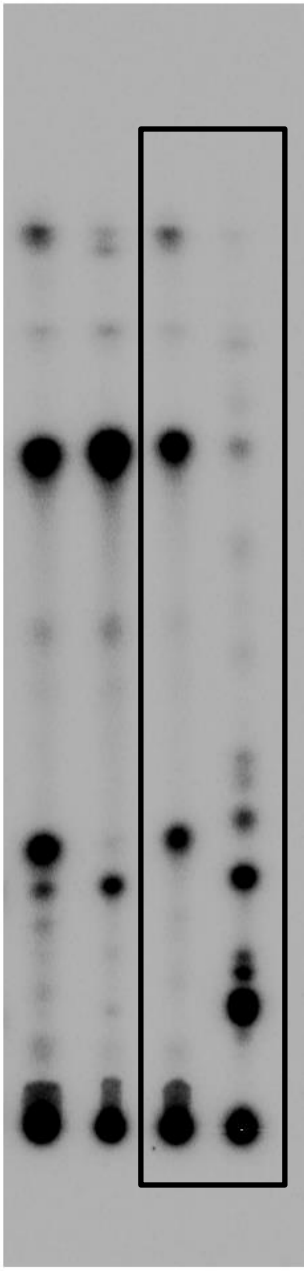
Resistant

Susceptible

Resistant

Supplementary Figure 23. Schematic indicating how antibiotic-resistant subpopulations can lead to unexplained clinical treatment failure. Graphic showing how antibiotic resistant subpopulations that are undetected by currently used diagnostic tests, such as that described in R/S-lo, can cause unexplained antibiotic treatment failure.





Supplementary Figure 24. Raw image files of lipid A thin layer chromatography.