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The Biology of Colistin Resistance: A Heteroresistance Mechanism and Inhibition of a Resistance Pathway

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Abstract

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By Emily K. Crispell

Antibiotic resistant infections are a significant and increasing cause of morbidity and mortality worldwide. In particular, infections caused by multi-drug resistant Gram negative bacteria recalcitrant to commonly used classes of antibiotics are of particular concern, as the antibiotic colistin is often the only remaining treatment option. Given the importance of this last-resort antibiotic, it is imperative that we fully understand the scope of colistin resistance mechanisms so that novel treatment strategies can be identified. In this work, we utilized an *Enterobacter cloacae* model of colistin heteroresistance to gain insight into this elusive resistance mechanism. The heteroresistant strain harbored both colistin resistant and susceptible subpopulations that were genetically indistinguishable yet transcriptionally distinct. The resistant subpopulation increased during antibiotic treatment, receded to baseline after subculture without drug, and was distinct from persisters. Presence of the resistant subpopulation and modification of colistin drug target lipid A was dependent on the histidine kinase gene *phoQ*. Colistin therapy failed to rescue mice infected with the heteroresistant strain, however treatment of mice infected with the *phoQ* mutant was successful, indicating that resistant bacterial subpopulations can cause antibiotic treatment failures. As a further approach to reduce the impacts of colistin resistance, we utilized a model of colistin resistant *Acinetobacter baumannii* to develop a resistance inhibition strategy. We identified a small molecule inhibitor of the *naxD*-controlled colistin resistance lipid A modification pathway via tandem *in vitro* and *in silico* screens. The inhibitor restored colistin susceptibility in a *naxD*-dependent manner and blocked the addition of galactosamine onto lipid A. Finally, the inhibitor reduced colistin resistance in a panel of clinical *A. baumannii* isolates, highlighting the potential for modulation of resistance expression to restore colistin efficacy for diverse strains. Altogether, these results provide fundamental insights into the biology of colistin resistance, and set the stage for continued development of novel therapeutics to combat antibiotic resistance in the clinic.

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CHAPTER 1: Introduction

Overview of the Antibiotic Resistance Problem

Antibiotic resistant infections are a growing problem that threaten the healthcare and well-being of patients worldwide. In 2013 alone, the CDC estimated that over 2 million cases and 23,000 deaths were directly attributed to antimicrobial resistant infections in the United States (1). Furthermore, the estimated yearly worldwide death rate due antibiotic resistance is predicted to increase from 700,000 associated deaths in 2014 to 10 million deaths annually by 2050 if left unchecked, surpassing even the death rate due to cancer (2). In 2008, the Infectious Diseases Society of America highlighted an important group of bacterial organisms, collectively identified as the "ESKAPE" pathogens, that are in part a source for this resistance increase (3). Bacterial species that comprise the ESKAPE group include *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* assorted *Enterobacter* species that include *Enterobacter cloacae,* and *Escherichia coli*. Resistant infections caused by these bacterial species, particularly *Acinetobacter baumannii* and *Enterobacter spp.*, are associated with high mortality rates and increased length of hospital stays (4-6).

The emergence of antibiotic resistance is not a new phenomenon. Indeed, Nobel Prize winning scientist Sir Alexander Fleming noted resistance to the antimicrobial penicillin as early as 1929, later determined by others to be the result of a secreted enzyme (7, 8). This observation occurred simultaneously with Fleming's initial discovery that the penicillin compound produced by the mold *Penicillium notatum* demonstrated growth inhibitory activity against *Staphylococcus aureus* (8), and preceded the largescale production of penicillin for clinical use as an antimicrobial therapy in 1940 by co-Nobel Laureates Sir Howard Florey and Ernst Chain (9). A substantial portion of the antibiotics utilized clinically in the early years of antimicrobial treatment availability are naturally produced by microorganisms, therefore innate resistance mechanisms to these

antibiotics exist to protect the producer species. Such naturally produced early antibiotics included the tetracyclines, streptomycin, and chloramphenicol (10). The discovery of the tetracycline class of antibiotics was aided by their natural production from the soil-dwelling organisms *Streptomyces aureofaciens* and *Streptomyces rimosus* (11, 12). *S. rimosus* tetracycline producer strains encode tetracycline resistance genes *tetA* and *tetB* that function by transporting the antibiotic outside of the cell via efflux to prevent antibiotic activity (13). *Streptomyces griseus,* the producer organism of streptomycin, uses the enzyme AphE to phosphorylate streptomycin, thereby abrogating antimicrobial action within the producer cell (14).

Evolution of Antibiotic Resistance

Antibiotic resistance can naturally develop in susceptible bacteria as a result of the strong selective pressures for survival that are imposed during antibiotic exposure. The transmission of resistance mechanisms from producer or other resistant species along with the evolution of new resistance mechanisms that arise during antibiotic treatment have both contributed to the acquisition and spread of antimicrobial resistance. The transfer of resistance amongst different organisms is mediated by the exchange of genetic materials between each bacterium, and is supported by high genetic similarities of resistance genes found throughout diverse bacterial species (15, 16). Such transfers may originate from the packaging and transport of genetic materials by bacteriophages, through DNA exchange by direct conjugation between bacterial cells, or through the uptake of extracellular DNA via cellular competence. Multiple studies have identified antibiotic resistance markers in the genetic content of naturally occurring bacteriophages; such phages were isolated from a variety of environments, including raw poultry, fertilized soil, and clinical sources (17-19). Similarly, naturally occurring plasmids that encode

antibiotic resistance markers have been identified from diverse bacterial species over the last several decades (20-22). Conjugation and plasmid transfer was identified as a contributing factor to dissemination of the *vanA* vancomycin resistance gene amongst *Enterococcus faecium* strains (23, 24). More recently, the colistin resistance gene *mcr-1* was identified on plasmids isolated from poultry and clinical samples worldwide, supporting widespread dissemination of this resistance gene and raising concerns for the continued efficacy of this so-called "last resort" antibiotic (25, 26). Direct transfer of resistance genes via plasmid conjugation between the unrelated species *Enterococcus faecalis* and *Escherichia coli* was demonstrated in gnotobiotic mice (27). Such cross-species genetic transfer and the uptake of resistance genes has also been demonstrated to occur via natural competence, as with *Acinetobacter baylyi,* an organism closely related to the ESKAPE pathogen *Acinetobacter baumannii* (28). Genetic exchange in this system was dependent on the lysis of neighboring *Escherichia coli* cells. Extracellular DNA released upon the killing of *E. coli* was implicated as the primary source of exchanged genetic material, as DNase treatment inhibited resistance transfer (28). Competence and the intraspecies exchange of genetic material is also thought to play an important role in the evolution of resistance for naturally competent organisms, such as *Neisseria meningitidis* and *Streptococcus pneumoniae* (29, 30).

In addition to acquisition of foreign resistance genes, new antibiotic resistance mechanisms can also evolve innately during antibiotic exposure. Strong selective pressures for survival due to the lethality of antibiotic action are imposed on bacteria during antibiotic treatment. Therefore, genetic mutations that confer fitness advantages will be selected. The evolution of both vancomycin and daptomycin resistance during antibiotic therapy was observed in studies that tracked the clinical progression of disease in *Staphylococcus aureus* infected patients, and whole genome sequencing of isolates allowed for identification of genetic mutations that supported these

increases in resistance (31, 32). In another example, nonsynonymous mutations in the genetic sequences of penicillin binding proteins that support cell wall synthesis have been linked to the reduced efficacy of ß-lactam antibiotics in a variety of bacterial species isolated from different patients (33-36). Outside the clinic, environmental sources of antibiotic exposure play an important role in the evolution of antibiotic resistance, as antibiotics in current clinical usage have been detected in sub-inhibitory concentrations in samples collected from soil, ground water, and drinking water (37). Importantly, these low level antibiotic exposures can still confer enough selective pressure for resistance to arise (38, 39).

Beyond the classical resistance mechanisms that rely on genetic changes, bacteria are also capable of evading antibiotic mediated killing through a decreased growth phenotype, termed "persistence". The persistence phenomenon was described soon after the introduction of antibiotics for clinical therapy, as penicillin treatment of Staphylococcal cultures inoculated into broth or serum frequently resulted in a small proportion of survivor cells (40). This subpopulation of persister cells was hypothesized to circumvent antibiotic action by remaining in a non-growing dormant state, as penicillin was observed to exert highest activity on cells in the logarithmic phase of growth. Furthermore, the progeny of persister cells were no more antibiotic resistant than the initial parental population, indicating that the resistance state was unstable. In additional support of this naturally occurring dormancy-dependent phenotype, manipulation of culture conditions to reduce population growth rates also decreased penicillin mediated killing. Indeed, single cell-microscopy later visually demonstrated that large populations of bacterial cells inherently contain subpopulations of non-growing or slowly growing cells (41). Since the initial discovery of persistence, additional studies have demonstrated that cells with reduced metabolic output are broadly tolerant to multiple classes of antibiotics (42-44). Furthermore, reservoirs of slow and non-

growing persister cells were the primary source of infection relapse following antibiotic therapy in a mouse model of *Salmonella* infection, indicating that persistence might be a source of antibiotic tolerance in the clinic (45).

Colistin as a last-line therapy

In recent years, increases in resistance to multiple classes of antibiotics amongst Gram negative bacterial infections, including those caused by *Acinetobacter baumannii*, *Escherichia coli, Klebsiella pneumoniae,* and *Enterobacter cloacae,* have led to the reintroduction of colistin usage in the clinic (1, 46-51). Colistin, also referred to as polymyxin E, is a polypeptide antibiotic of the polymyxin class of antimicrobials that is non-ribosomally synthesized by the soil bacterium *Bacillus polymyxa* (52, 53). It was introduced to the clinic during the 1960s, but fell out of favor due the development of newer antibiotics with less toxic side effects (54). Colistin may be administered in the salt form as colistin sulfate or as the prodrug colistin methanesulfonate (55). The structure of colistin consists of a decapeptide chain and ring containing 6 cationic residues attached to a fatty acid tail. These structural properties help target colistin to the outer cell membrane of Gram negative bacteria (53). The mechanism of action is not fully understood, but is thought to consist of initial electrostatic interaction between the cationic amino acids of colistin with the negatively charged phosphate moieties on the lipid A molecule on the outer membrane (56). This interaction causes displacement of cations associated with the outer membrane, leading to membrane insertion and disruption of both the outer and inner cell membranes (56). This model of activity is supported by fluorescence spectroscopy experiments that demonstrate membrane binding and leakage along with electron microscopy experiments demonstrating that colistin treatment induces shedding of the outer membrane and loss of cytoplasmic contents (57-59).

Reintroduction of colistin usage in the clinic has corresponded with reports of colistin resistance in bacterial isolates that are also multi-drug resistant (60-62). Historically, colistin resistance was associated with the acquisition of chromosomal mutations, however the first plasmid-mediated resistance mechanism was reported in 2016 (25). In accordance with other antibiotic producing bacteria, the native colistin producer species *Bacillus polymyxa* encodes an innate colistin resistance mechanism that synthesizes a colistinase enzyme to break down the antibiotic through cleavage of its polypeptide portion at the junction between the cyclic and linear peptide chain (63). Surprisingly, other reports of colistinase in bacteria of clinical importance have not been described (49). Instead, resistance primarily arises from mechanisms that modify the outer membrane drug target.

In order to understand the most common resistance mechanisms against colistin, one must first understand organization and structure of the Gram negative bacterial outer membrane. This complex structure is composed of an asymmetric bilayer, with a periplasmic-facing layer composed of phospholipids and the outward-facing layer composed predominately of lipid A (64). Lipid A is typically required for cell survival, although reports of non-essentiality do exist (65-67). The structure of lipid A is well characterized in *Escherichia coli,* and is typically composed of two phosphorylated glucosamine subunits attached to 6 hydrophobic acyl chains. The phosphate groups confer the negative charge associated with the cell surface. Lipid A serves as an anchor for the larger structural molecule lipopolysaccharide (LPS), which is composed of a lipid A base attached to a core anionic oligosaccharide and final outer oligosaccharide of variable length commonly referred to as O-antigen (68, 69). The lipid A synthetic pathway is well conserved amongst Gram negative bacteria, and requires a series of enzymatic steps that occur in the

cytoplasm to generate the mature core molecule, after which it is transferred to the periplasm for further modification before final trafficking to the outer membrane (70).

Colistin Resistance in the Clinic

The majority of described mechanisms for resistance to cationic antimicrobial peptides such as colistin result from lipid A modifications that mask the negative charge conferred by the phosphorylated lipid A base structure. Well characterized colistin resistance modifications that are described in multiple species of bacteria involve the covalent attachment of phosphoethanolamine or 4-amino-4-deoxy-L-arabinose (L-Ara4N) molecules to the phosphate moieties of the lipid A glucosamine subunits (71-75), however other covalent additions such as galactosamine and palmitate have also been reported (76-78). Regulation of these modifications is complex, but often relies on activation of two-component regulatory systems, such as those encoded by the *phoPQ* and *pmrAB* genes in *E. coli* and *Salmonella enterica,* in response to environmental sensing (79-82). These genetic loci encode membrane-associated sensor proteins (PhoQ or PmrB) that recognize a variety of environmental signals and translate those detection events to initiate a signaling cascade. Signaling is mediated via autophosphorylation of the sensor protein at a conserved histidine residue and subsequent phosphotransfer to an aspartic acid residue on the cognate response regulator (PhoP or PmrA, respectively), which then undergoes conformational rearrangement to bind DNA promoters and modulate downstream gene transcription (81, 83, 84). The regulatory networks of PhoPQ and PmrAB are complex and interrelated, as both systems participate in a positive biofeedback loop and PhoP activation can subsequently stabilize activation of PmrA via intermediate proteins in some species (85-87). Despite regulon complexities, lipid A modification genes such as the *arnBCADTEF* operon that catalyzes the L-ara4N addition and

pmrC that catalyzes phosphoethanolamine additions are both under the control of these networks (88-91). A variety of mutations that alter regulatory output through these two component systems and thereby increase resistance to cationic antimicrobials including colistin have been described. Such mutations include those that increase activation of signaling through the sensor protein (71, 92, 93) or response regulator (81, 94, 95) and also mutations that inactivate negative regulators of these systems, such as the protein MgrB (96, 97). Intriguingly, sRNAs that postranscriptionally regulate PhoPQ expression have been described, however mutations in these loci have not yet been linked to colistin resistance (98, 99).

Despite heightened awareness of antibiotic resistance and characterization of a broad range of resistance mechanisms, unexplained treatment failures still occur that cannot be attributed to known resistance mechanisms (100). This phenomenon is particularly worrisome due to the aforementioned increases in multi-drug resistant infections that already have few to no antibiotic options for treatment. One poorly defined resistance mechanism that could in part be responsible for these treatment failures is heteroresistance (101). Although a comprehensive definition of heteroresistance has been debated, it is largely defined as a bacterial strain that contains a subpopulation of antibiotic susceptible cells coexisting with another subpopulation that exhibits increased resistance (102). Likewise, the clinical relevance of heteroresistance is also debated, particularly in the historically recognized class of vancomycin heteroresistant *Staphylococcus aureus* (103, 104). Despite these uncertainties, heteroresistance has been identified against several classes of antibiotics, including colistin, and in various bacterial species of clinical relevance. Our lab was one of the first to identify colistin heteroresistance in the ESKAPE pathogen, *Enterobacter cloacae* (105). Likewise, heteroresistance to colistin and other antibiotics is also reported in the

multi-drug resistant ESKAPE pathogens *Acinetobacter baumannii* and *Klebsiella pneumoniae* (106-111).

The primary goal of this thesis was to enhance our understanding of the biology of colistin resistance by both exploring the phenomenon of colistin heteroresistance and utilizing genetic information to manipulate the expression of colistin resistance. In Chapter 2, we further define the phenomenon of heteroresistance by investigating the genes and molecular mechanisms that mediate the colistin heteroresistant phenotype. Identification of genetic systems that contribute to heteroresistance is critical for defining regulatory mechanisms that modulate heterogeneous expression of antibiotic resistance, and also for uncovering genetic targets that could serve a role in future development of new antimicrobial inhibitors. In Chapter 3, we present a new strategy to modulate the expression of colistin resistance through inhibition of a resistance modification pathway in an effort to restore efficacy of this last resort drug. Overall, this work broadens our understanding of a poorly characterized antimicrobial resistance phenomenon and provides hope for reversing antibiotic resistance in an era of declining antibiotic efficacy.

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CHAPTER 2: Antibiotic Failure Mediated by a Resistant Subpopulation in *Enterobacter cloacae*

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

by

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analysis was performed by EKC, GKT, KV, TDR, and SB. JP synthesized and purified host antimicrobials. Study was planned and directed by DSW.

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Abstract

Antibiotic resistance is a major public health threat, further complicated by unexplained treatment failures caused by bacteria that appear antibiotic susceptible. We describe an *Enterobacter cloacae* isolate harboring a minor subpopulation highly resistant to the last-line antibiotic colistin. This subpopulation was distinct from persisters, became predominant in colistin, returned to baseline after colistin removal, and was dependent on the histidine kinase PhoQ. During murine infection, but in the absence of colistin, innate immune defenses led to an increased frequency of the resistant subpopulation, leading to inefficacy of subsequent colistin therapy. An isolate with a lower frequency colistin-resistant subpopulation similarly caused treatment failure but was misclassified as susceptible by current diagnostics once cultured outside the host. These data demonstrate the ability of low frequency bacterial subpopulations to contribute to clinically relevant antibiotic resistance, elucidating an enigmatic cause of antibiotic treatment failure and highlighting the critical need for more sensitive diagnostics.

Introduction

Antibiotic resistance threatens the delivery of safe and effective healthcare[1] and is projected to lead to 10 million annual deaths worldwide by 2050[2]. Failure of antibiotic treatment results in increased length of patient stay, healthcare costs and mortality[2]. Multi-drug resistant *Enterobacter* spp. have emerged as an increasing cause of hospital acquired infections[3-5], with the drug colistin being relied on as a last line treatment[6,7]. However, colistin resistant strains have emerged, further limiting treatment options[8]. Further complicating the treatment of some bacterial infections is the failure of antibiotic therapy in strains that are classified as susceptible; these infections may be non-responsive to treatment in ~10% of cases[9]. While relatively little is known about the causes of treatment failures, we show here that they can be mediated by antibiotic resistant subpopulations in *Enterobacter cloacae*. Furthermore, such antibiotic resistant subpopulations can be undetectable by current diagnostic tests.

Results

Phenotypically resistant subpopulation. A strain of *Enterobacter cloacae* was isolated from a renal transplant recipient[10] and was observed to harbor a distinct subpopulation with resistance to colistin, visualized as numerous colonies within the zone of inhibition upon testing by colistin Etest (we refer to the strain as "R/S", to indicate the presence of both resistant and susceptible subpopulations) (**Fig 1a)**. This was not observed with either colistin susceptible or resistant (**Supplementary Figure 1)** clinical strains. Population analysis profile (PAP) of R/S, in which a strain is assayed for survival on agar plates with increasing amounts of antibiotics, revealed a major proportion of bacteria (>90%) susceptible to 1 μg/mL colistin, and a highly resistant subpopulation, able to withstand at least 500 μg/mL colistin **(Fig 1b)**. This was in contrast to the susceptible strain that was uniformly killed by 1 μg/mL colistin, and the resistant strain that was uniformly killed by 200 μg/mL colistin. The proportion of the R/S colistin resistant subpopulation was increased to upwards of 80% upon exposure to colistin **(Fig 1c)**. Further analysis revealed that this increase was due to an initial selection against the colistin susceptible population over the first 2 hours of antibiotic exposure, followed by robust replication and expansion of the resistant population in the presence of the drug **(Fig 1d)**. Importantly, this suggests that the resistant cells are not persisters, which do not significantly expand in number during antibiotic treatment[11-13]. The increase in the resistant subpopulation was reversible, as subsequent growth after subculture in antibiotic free media led to a return of these cells to pre-treatment levels **(Fig 1c)**. This suggests that the resistant subpopulation is not the result of a stable mutation. Furthermore, bacteria from within the zone of inhibition (where antibiotic levels are high) and outside this region (where antibiotic is low or not present) on a colistin Etest plate **(Fig 1a)** exhibited identical levels of

susceptible and resistant populations after serial culturing in the absence or presence of colistin **(Supplementary Figure 2)**, suggesting that bacteria from these two growth conditions are identical. Indeed, deep sequencing of R/S grown with and without colistin (conditions in which the resistant population accounted for the vast majority or minority of the total population, respectively, as summarized in Supplementary Figure 3) revealed identical genomes. Taken together, these data show that a minor antibiotic resistant subpopulation is capable of replicating in the presence of antibiotic, becoming predominant, and mediating resistance to high levels of drug.

To determine whether the increase in the proportion of the resistant subpopulation occurs during antibiotic treatment *in vivo*, we infected mice with R/S and treated with colistin or PBS. In colistin treated mice, we observed a significant increase in the frequency of the resistant subpopulation of bacteria isolated from the peritoneum **(Fig 1e)** and liver **(Supplementary Figure 4)**. Surprisingly, there was also a robust increase in the resistant subpopulation during *in vivo* infection in the absence of colistin treatment **(Fig 1e, Supplementary Figure 4)**. By 48 hours, the percentage of the resistant subpopulation increased from <10% to >80% (**Supplementary Figure 5)**. These results highlight the process of infection as leading to a significant increase in the frequency of an antibiotic resistant subpopulation of bacteria.

Resistance to innate immune defenses. Various host pressures could be responsible for the increase in the colistin resistant subpopulation during infection. As macrophages are a major component of the early immune response[14], we tested their role by depleting these cells with clodronate liposomes[15] **(Supplementary Figure 6)** and subsequently infecting mice with R/S. In contrast to bacteria recovered from mice treated with control liposomes, which demonstrated a

robust increase in the frequency of the resistant subpopulation, those recovered from macrophage-depleted mice showed no such increase **(Fig 2a)**. Based on these results, we next determined whether macrophages were sufficient to cause the increase in the resistant subpopulation, by infecting them *in vitro*. During macrophage infection, the colistin resistant subpopulation increased to 40% within only 2 hours **(Fig 2b)**. Furthermore, this rise was dependent on internalization of the bacteria, since preventing phagocytosis with cytochalasin D abrogated this phenomenon **(Fig 2b)**. Therefore, macrophages are both required and sufficient for the increased frequency of the resistant subpopulation during infection, underlining a role for a specific innate immune cell type in this process.

Macrophages possess many antibacterials[16] and we hypothesized that specific components would be required for the increase in the frequency of the resistant subpopulation, testing reactive oxygen species (formed after treatment with hydrogen peroxide), lysozyme, and the murine cationic antimicrobial peptide CRAMP. All of these antibacterials resulted in a dose-dependent increase in the frequency of the colistin resistant subpopulation *in vitro* **(Fig 2c-e)**, as did LL-37, the human ortholog of CRAMP **(Supplementary Figure 7)**. These results led us to test whether the antibacterials were responsible for the increase in the resistant subpopulation during *in vivo* infection. We infected wild-type and triple knockout (TKO) mice lacking a functional NADPH oxidase (which leads to the production of reactive oxygen species[17]), lysozyme, and CRAMP. TKO mice were more susceptible to infection by R/S as they harbored over 10-fold more bacteria compared to WT **(Supplementary Figure 8)**, demonstrating the importance of these antimicrobials in host defense. While a robust increase in the frequency of the resistant subpopulation was observed in wild-type mice, this was abrogated in TKO mice **(Fig 2f)**. The frequency of the resistant subpopulation in mice lacking one of these three antimicrobials was not

significantly different from that in wild-type mice, while it was decreased in double KO mice lacking the NADPH oxidase and CRAMP or lysozyme **(Supplementary Figure 9)**. These data identify a role for specific host innate immune antibacterials in the increase of an antibiotic resistant subpopulation during *in vivo* infection.

Subpopulation-mediated antibiotic failure. To determine the relevance of the increase in frequency of the resistant subpopulation during *in vivo* infection, we tested whether the R/S strain was able to resist colistin treatment. We infected mice with either R/S or a colistin susceptible strain, and treated the mice with PBS (as a control) or high doses of colistin after establishment of infection to simulate the progression of infection and treatment in the clinic. The levels of the susceptible strain in the peritoneum **(Fig 3a)** and liver **(Fig 3b)** were significantly reduced by colistin treatment. In contrast, the R/S strain was refractory to treatment with colistin as its levels were unchanged between the treated and untreated groups **(Fig 3a-b)**. In a timecourse experiment, the level of the susceptible strain was reduced by 3 logs at 42 hours, whereas the level of the R/S strain was not diminished by colistin treatment, but instead increased by roughly 10-fold **(Supplementary Figure 10)**. These data demonstrate that the presence of the resistant subpopulation results in inefficacy of colistin to reduce bacterial levels *in vivo*. Further, these results provide *in vivo* evidence that the resistant subpopulation does not behave like persisters which do not significantly expand in number during antibiotic treatment.

We next tested whether the role of the host immune system in the increase of the resistant subpopulation was directly responsible for the inefficacy of antibiotic therapy. We first found that colistin treatment of R/S-infected mice could cause a significant reduction in bacterial levels if initiated at the time of infection (prior to the increase in the frequency of the resistant

subpopulation), but not if it was delayed until only 4 hours after infection **(Fig 3c)**. However, in macrophage-depleted mice, treatment with colistin at 4 hours became effective, leading to a reduction in bacterial levels **(Fig 3c)** and indicating that the host-driven increase in the frequency of the resistant subpopulation is responsible for the inefficacy of antibiotic treatment.

To further test the relevance of this *in vivo* phenomenon, we infected mice with a lethal dose of bacteria and treated with either PBS or colistin after 12 hours. Both the susceptible and R/S strains led to lethal infections in the absence of colistin **(Fig 3d,e)**. In the presence of colistin, only mice infected with the susceptible strain were rescued **(Fig 3d)**, whereas those infected with R/S still succumbed to infection within 100 hours **(Fig 3e)**. These data demonstrate the impact of an antibiotic resistant subpopulation in mediating a lethal infection in the presence of high dose antibiotic treatment.

PhoQ-dependent resistant subpopulation. We next set out to determine the molecular mechanism underlying the phenotype of the resistant subpopulation. RNAseq analysis was conducted **(Supplementary Figure 3, Table S1)** to determine whether there were transcriptional differences between the susceptible and resistant subpopulations of R/S. In total this analysis revealed 325 genes upregulated and 360 genes downregulated in the resistant subpopulation as compared to the susceptible subpopulation **(Table S2)**. While this approach should detect differences between the two subpopulations, it may also identify expression differences due to colistin treatment. Among the upregulated genes, we noticed a signature **(Table S3)** associated with the two-component histidine kinase PhoQ[18-25], which has previously been implicated in polymyxin resistance, in part through its role in modification of the lipid A portion of lipopolysaccharide[26]. To validate the RNAseq data, we confirmed that the resistant subpopulation

expressed higher levels of the predicted lipid A modification genes *arnB* and *eptA*[27] **(Supplementary Figure 11)**. These data suggested that R/S displayed a modified lipid A profile, which we confirmed by thin layer chromatography (TLC) **(Supplementary Figure 12)**. Further, modified lipid A species increased in abundance during growth of R/S in the presence of colistin, consistent with their expression by the resistant subpopulation **(Supplementary Figure 12)**. To test whether the lipid A modifications were dependent on PhoQ, we constructed an R/S deletion mutant lacking *phoQ* (Δ*phoQ*). Indeed, lipid A from the Δ*phoQ* strain lacked the specific lipid A modifications observed in wild-type R/S that were enhanced in the presence of colistin, which were restored in a *phoQ* complemented strain **(Supplementary Figure 12)**. Thus, the R/S resistant subpopulation exhibits PhoQ-dependent lipid A modifications and is transcriptionally distinct when compared to the susceptible subpopulation.

To interrogate the potential contribution of PhoQ to the R/S resistance phenotype, we examined the colistin resistance profile of Δ*phoQ*. Strikingly, the Δ*phoQ* strain exhibited a complete absence of the resistant subpopulation by Etest, while the susceptible subpopulation remained unaffected, as the border of the zone of clearing was unaltered from that of wild-type R/S **(Fig 4a)**. Complementation with *phoQ* restored the presence of the resistant subpopulation **(Fig 4a)**. This was also confirmed by PAP, where Δ*phoQ* lacked the resistant subpopulation present in R/S and behaved similar to the susceptible strain **(Fig 4b)**. Importantly, R/S and Δ*phoQ* harbored equivalent levels of persisters, clearly indicating that the colistin resistant subpopulation (which depends on PhoQ) is not made up of persister cells **(Supplementary Figure 13)**. The *phoQ* mutant additionally exhibited no colistin resistant subpopulation after exposure to host antimicrobials **(Supplementary Figure 14A)**, during macrophage infection **(Supplementary Figure 14B)**, or during *in vivo* infection **(Supplementary Figure 14C)**. Without the presence of

the resistant subpopulation, Δ*phoQ* was susceptible to colistin treatment *in vivo*, exhibiting a significantly decreased bacterial load **(Fig 4c)**. Furthermore, the ability of colistin to rescue mice from an otherwise lethal inoculum was restored during infection with Δ*phoQ* **(Fig 4d)**. Thus, the presence of the colistin resistant subpopulation is dependent on PhoQ, which is required for a lethal drug resistant infection.

Undetected subpopulation. The size of the resistant subpopulation can vary greatly between strains, as exemplified by a distinct *E. cloacae* clinical isolate (termed R/S-lo) which harbors a colistin resistant subpopulation between 0.01 and 0.001% of the total population **(Fig 5a)**, over 1,000-fold less prevalent than that of R/S when grown in media without antibiotic. Similar to R/S, the increase of the R/S-lo resistant subpopulation in the presence of colistin **(Supplementary Figure 15)** was due to initial selection against the susceptible subpopulation followed by expansion of the resistant subpopulation **(Supplementary Figure 16)**. The frequency of the resistant subpopulation was likewise increased by treatment with H_2O_2, lysozyme, CRAMP, and LL-37 **(Supplementary Figure 17)**, during macrophage infection **(Supplementary Figure 18)**, and during *in vivo* infection of mice **(Supplementary Figure 19)**, and was greatly diminished in macrophage-depleted **(Supplementary Figure 20)** and TKO mice **(Supplementary Figure 21)**. These data revealed that similar to R/S, the frequency of the resistant subpopulation of R/S-lo is increased by colistin as well as the activity of specific host innate immune components. During *in vivo* infection, while the levels of a susceptible strain were significantly reduced by colistin treatment, the levels of R/S-lo were unaffected **(Supplementary Figure 22)**. These data directly correlated with a failure of colistin therapy to rescue R/S-lo infected mice from a lethal infection **(Fig 5c)**, whereas mice infected with a susceptible strain were completely rescued **(Fig 5b)**.

Importantly, unlike R/S, R/S-lo was clinically classified as being susceptible to colistin, as the resistant subpopulation (present at a frequency of only 1 in 10,000 CFU) was not detected by Etest **(Fig 5d)**. Therefore, this seemingly colistin susceptible strain, harboring an undetected resistant subpopulation, is capable of causing an antibiotic resistant and lethal infection *in vivo*.

It is worrisome that R/S-lo was not identified as colistin resistant, and we wondered whether the resistant population could be detected by diagnostic testing when it is more frequent during host infection. We directly plated peritoneal lavage samples from infected mice in the absence of subculture, and were able to detect the R/S-lo resistant subpopulation by Etest, as indicated by colonies within the zone of inhibition **(Fig 5e, g)**. In contrast, when these samples were processed by the clinical microbiology laboratory (as would occur with a sample from a human patient, and including a critical subculture step), Etest could no longer detect the diminished resistant subpopulation **(Fig 5f, g)**. Strikingly, these data reveal how and when detection of the resistant subpopulation can be missed during routine diagnostic testing, and how this can translate into an unexplained failure of antibiotic therapy.

Discussion

The findings presented here highlight the role of a minor colistin resistant bacterial subpopulation in mediating antibiotic treatment failure *in vivo*. This resistant subpopulation is genetically identical to the susceptible subpopulation, but exhibits differences in gene expression and lipid A modification. Furthermore, the presence of this resistant subpopulation is dependent on the histidine kinase PhoQ. The data also highlight an unexpected role for specific host innate immune components (lysozyme, CRAMP and H_2O_2) in the increase of the antibiotic resistant subpopulation during infection. The increase in the frequency of the resistant subpopulation

induced by host immune pressure *in vivo* was shown to be critical for eventual failure of colistin therapy.

Like bacterial persistence, the phenotypic resistance phenomenon we describe involves a resistant subpopulation, but there are important differences. Persistence involves a small subpopulation of bacteria that are tolerant to a drug due to a state of low metabolic activity, with no or limited replication[28]. Wakamoto et al showed that in some cases persisters can replicate, although it is at a very low rate, and is insufficient to cause an overall increase in the numbers of the population[11]. In contrast, we describe a resistant subpopulation that rapidly replicates both *in vitro* and *in vivo* in the presence of antibiotic, and leads to a very significant overall increase in bacterial population level **(Fig 1d, Supplementary Figure 10)**. Further, we directly show that the PhoQ-dependent colistin resistant subpopulation is distinct from persisters, which are also present but independent of PhoQ **(Supplementary Figure 13)**. Several papers have recently demonstrated the importance of persisters as a reservoir of infection during antibiotic treatment *in vivo*[12], which can continue to replicate after treatment has been stopped, leading to relapse[13]. In contrast, we demonstrate that the colistin resistant subpopulation described here facilitates bacterial growth and subsequent host lethality even in the presence of antibiotic. Persistence has also been linked to immune pressure, as bacteria within macrophages can have increased numbers of persisters[29]. We observe a similar link, as both *in vitro* and *in vivo,* specific host antimicrobials lead to an increased frequency of the resistant subpopulation. Taken together, both persisters and the resistant subpopulation described here highlight the ability of a minority of a bacterial population to exert a striking effect on the outcome of infection and antibiotic treatment. While persisters are kept at bay by antibiotic treatment and form a reservoir that can cause relapse, the colistin resistant

subpopulation described here has the ability to cause acute infection and lethality during the course of antibiotic treatment.

We propose to refer to the resistance phenomenon described here as clonal heteroresistance. The phenomenon of heteroresistance, in which a resistant subpopulation exhibits an increased level of antibiotic resistance relative to the larger susceptible subpopulation, was described as far back as 1947[30]. However, its relevance to infection and resistance has remained unclear, and even its definition has been debated. We use the term clonal heteroresistance to distinguish the phenomenon we describe from the blanket term heteroresistance which is often used to refer to mixed populations of genetically distinct bacteria[31-34]. We show that clonal heteroresistance, in addition to mediating lethal infection in the presence of antibiotic, can also go undetected and cause unexplained treatment failure during *in vivo* infection **(Supplementary Figure 23)**. Current widely used methods of antibiotic susceptibility testing rely on *in vitro* culture and analysis. Our data show that these methods can greatly alter results and present an inaccurate picture of the level of *in vivo* resistance. Our findings highlight both a need and opportunity for improved diagnostics to detect antibiotic resistant subpopulations and ultimately prevent such treatment failures.

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Competing Financial Interests

The authors declare no competing financial interests.

Figures

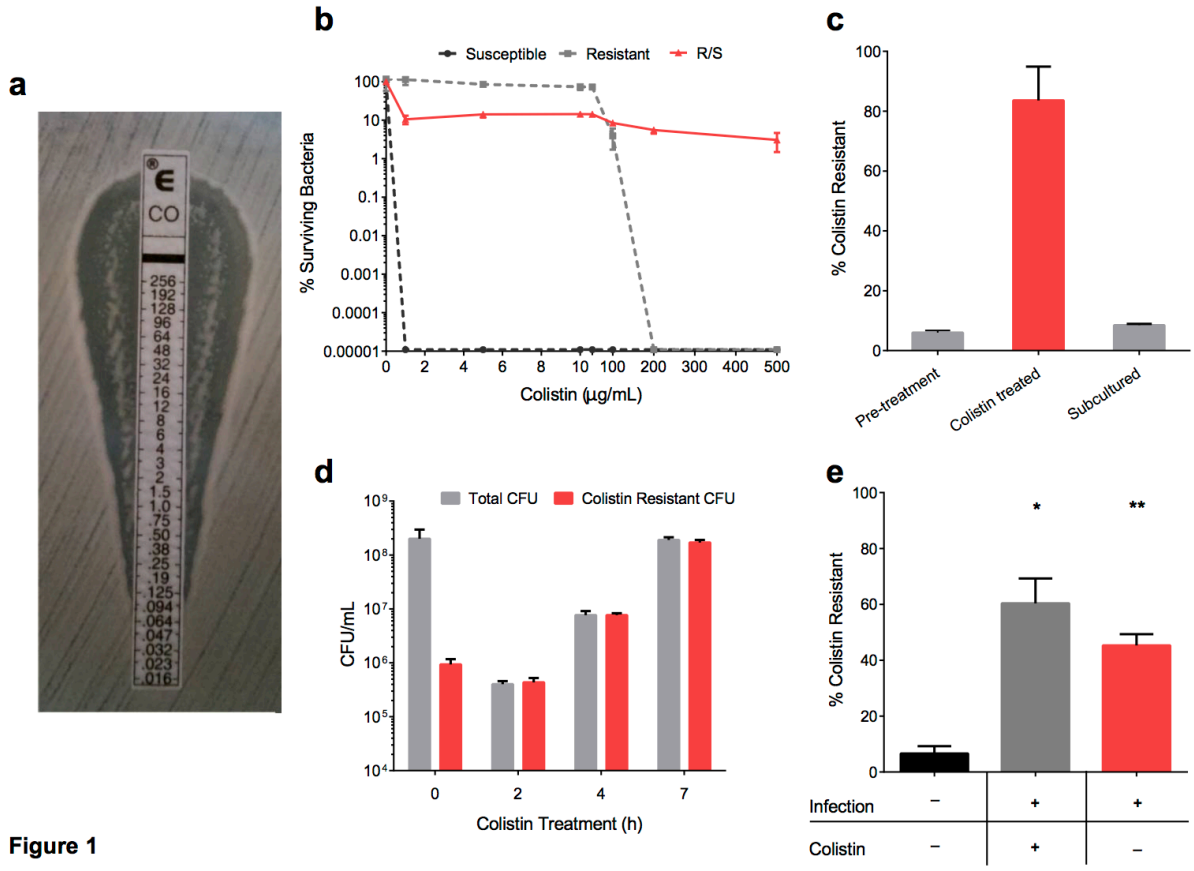

Figure 1

Figure 1. A colistin resistant subpopulation increases in frequency during *in vivo* infection

. a, Testing of *E. cloacae* clinical isolate R/S by colistin Etest, with drug concentration indicated in µg/mL. Colonies within the zone of inhibition indicate a colistin resistant subpopulation. Data is representative of >10 Etests. **b,** Population analysis profile of R/S as well as colistin-susceptible and -resistant *E. cloacae* clinical isolates (n=3). **c,** Percentage of the colistin resistant subpopulation in R/S in antibiotic-free media, after 24 h treatment with 100 µg/mL colistin, and after 8 h subculture of the colistin treated culture in antibiotic free media. "% Colistin resistant" represents the number of CFU in each culture that can grow on media containing 100 µg/mL

colistin, as a percentage of the total CFU in the culture (n=3). **d,** Colistin resistant and total CFU of R/S during 7 h treatment with 100 μg/mL colistin in liquid culture (n=3). **e,** Pre-infection inoculum (black bar) was used to infect mice, and peritoneal lavage was performed and harvested 24 h later and plated to calculate % colistin resistant CFU (n=5). Mice were treated at 8, 14 and 20 h with colistin (grey bar) or PBS (red bar). Error bars represent s.e.m. (Mann-Whitney test, * $p < 0.05$, ** $p < 0.01$).

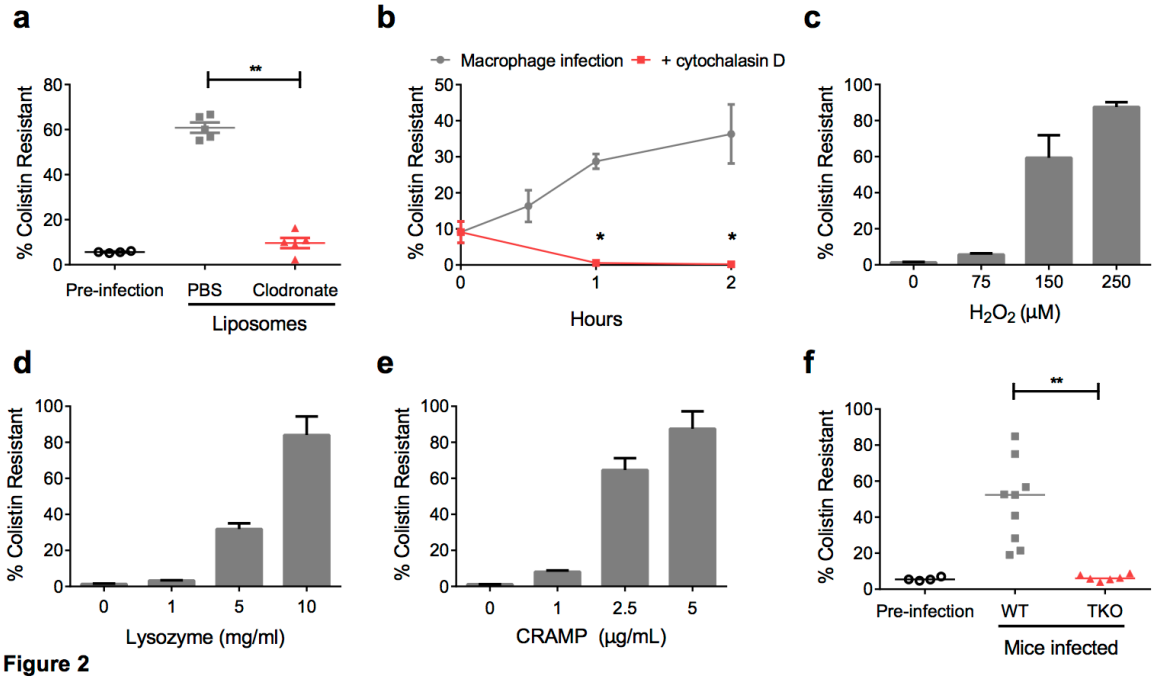

Figure 2

Figure 2. Innate immune host defenses are required for the increased frequency of the colistin resistant subpopulation during infection.

a, Mice pre-treated with PBS liposomes (as a control; grey) or clodronate liposomes (to deplete macrophages; red) were infected with R/S (pre-infection; black). After 8 h, peritoneal lavage fluid was harvested and plated to calculate % colistin resistance (n=5). **b,** Murine bone marrow-derived macrophages were untreated or pretreated with cytochalasin D, infected with R/S, and % colistin resistance was calculated at the indicated timepoints (n=6). **c-e**, R/S was either untreated or treated with the indicated amounts of (**c**) H_2O_2, (**d**) lysozyme, or (**e**) CRAMP for 5 h, and % colistin resistance was calculated (n=3). **f,** Wild-type (WT; grey) or triple knockout (TKO; red) mice lacking the gp91 subunit of the NADPH oxidase, lysozyme, and CRAMP were infected with R/S (pre-infection; black). At 8 h postinfection, peritoneal lavage fluid was harvested and plated to calculate % colistin resistance (n=5). Data is compiled from two independent experiments. Error

bars represent s.e.m. (Mann-Whitney test, ** $p < 0.01$). in **a,f**, (Student's two-tailed t-test, * $p < 0.05$) in **b**.

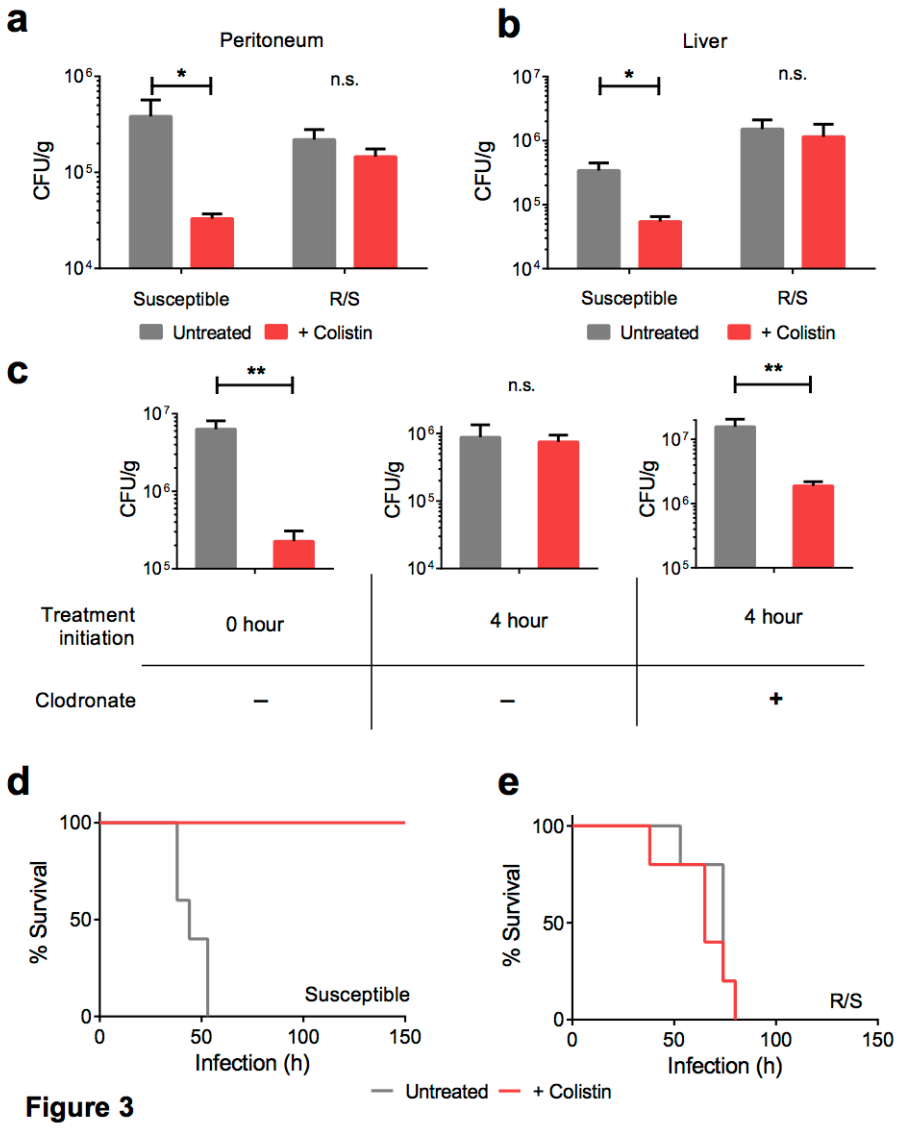

Figure 3

Figure 3. R/S is refractory to colistin during infection and leads to colistin treatment failure. **a,b,** Mice infected with R/S or the susceptible isolate were treated with colistin at 8, 14 and 20 h. CFU were quantified at 24 h in the (**a**) peritoneal lavage fluid and (**b**) liver (n=5). **c,** Mice pretreated with PBS (first and second panels) or clodronate (third panel) liposomes were infected with R/S and treated with colistin at 0 h (first panel) or 4 h (second and third panels). A second dose of

colistin was administered 2 h after the first, and 2 h later peritoneal lavage fluid was plated to enumerate CFU (n=5). **d,e,** Survival of mice infected with R/S or the colistin susceptible isolate. Mice were treated with colistin or PBS starting at 12 h post infection, with additional doses given every 6 h thereafter. Surviving mice were monitored until day 24 (n=5). Error bars represent s.e.m., center values represent median. (Mann-Whitney test, * $p < 0.05$, ** $p < 0.01$, n.s. = not significant).

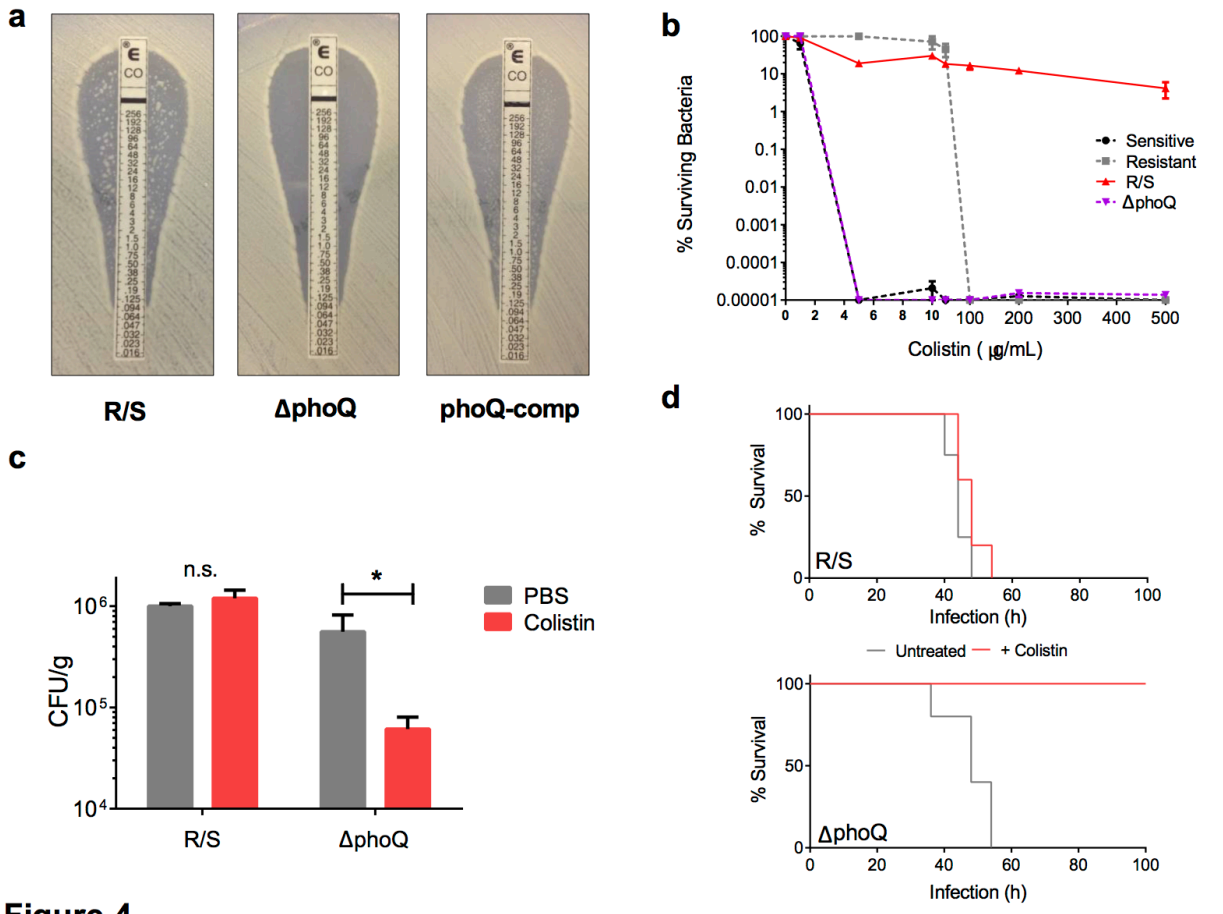

Figure 4

Figure 4. PhoQ is required for the presence of the colistin resistant subpopulation.

a, Colistin Etest of R/S, Δ*phoQ*, and the complement (phoQ-comp) strains, with drug concentration indicated in µg/mL. Colonies within the zone of inhibition indicate a colistin resistant subpopulation. Data are representative of 2 Etests. **b,** Population analysis profile of R/S, Δ*phoQ,* and colistin susceptible and resistant *E. cloacae* strains (n=3). **c,** Mice infected with R/S or Δ*phoQ* were treated with colistin at 8, 14 and 20 h. CFU were quantified at 24 h in the peritoneal lavage fluid (n=5). **d,** Survival of mice infected with R/S (upper panel) or Δ*phoQ* (lower panel). Mice were treated with colistin or PBS starting at 12 h post infection, with additional doses given

every 6 h thereafter (n=5). Error bars represent s.e.m., center values represent median. (Mann-Whitney test, * $p < 0.05$, n.s. = not significant).

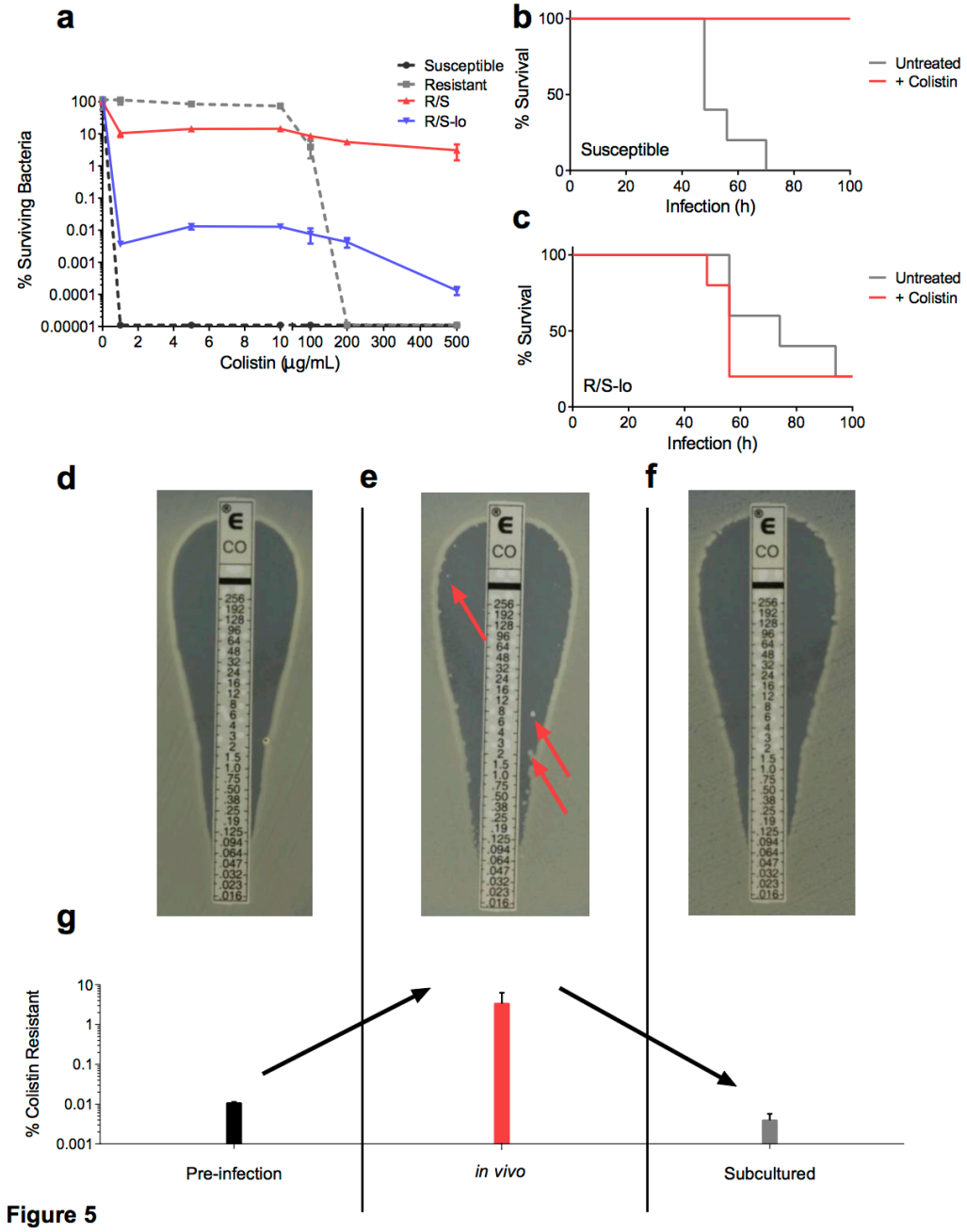

Figure 5

Figure 5. Clinical isolate harboring an undetected colistin resistant subpopulation causes a lethal, antibiotic resistant infection.

a, Population analysis profile of *E. cloacae* clinical isolate R/S-lo compared to R/S and the colistin susceptible and resistant isolates (n=3). **b-c,** Infection of mice with (**b**) the colistin susceptible isolate or (**c**) R/S-lo with or without colistin treatment every 6 h and initiated 12 h post infection (n=5). Surviving mice were monitored until day 24. **d-f**, Colistin Etest, with drug concentration indicated in μg/mL, of R/S-lo from (**d**) pre-infection inoculum, (**e**) peritoneal lavage sample from a mouse infected for 8 h and (**f**) the peritoneal lavage sample subcultured overnight in drug-free media (n=5). Colonies in the zone of inhibition (**e**, red arrows) indicate resistant bacteria. Images representative of 5 individual samples are shown. **g**, The samples from **d-f** were plated to determine % colistin resistance (n=5). Error bars represent s.e.m.

Methods

Bacterial strains. *E. cloacae* strain R/S was isolated from a blood sample from a renal transplant recipient at Emory University Hospital (Atlanta, GA). *E. cloacae* R/S-lo, the colistin susceptible strain Mu819, and the colistin resistant strain Mu117 were isolated from urine samples from patients at other Atlanta, GA hospitals.

Bacterial culture. All bacterial strains were streaked on Mueller-Hinton (MH) agar plates and grown in MH medium at 37°C in a shaking incubator from a single colony before each experiment. Colony forming units (CFU) were determined by plating dilutions on MH agar plates incubated at 37°C and then counting bacterial colonies at the lowest distinguishable dilution.

Bacterial genetics. To generate strain Δ*phoQ,* 600-700bp upstream and downstream fragments of the genomic region surrounding *phoQ* were PCR amplified with primers 81 and 118, and 82 and 119, respectively **(Table S4)** and fused with the hygromycin resistance cassette HmR amplified from vector pMQ310 with primers 79 and 80[35] using SOE PCR[36]. The suicide vector pEXR6K was generated by replacing the pMB1 ori from PCR linearized plasmid pEX100T[37] using primers 110 and 111 with the R6K ori amplified from plasmid pMQ310 with primers 108 and 109 using the Gibson Assembly Cloning Kit (Invitrogen). The HmR construct was inserted into SmaI (New England Biolabs) digested pEXR6K by Gibson assembly and the resulting plasmid was transformed to strain R/S by electroporation. Transformants were selected on MH agar containing 150μg/mL hygromycin (Sigma) then passaged to LB agar containing 20% sucrose and no NaCl to counterselect for vector loss. Chromosomal replacement of *phoQ* with the hygromycin marker was confirmed by Sanger sequencing. To generate strain *phoQ*-comp, the promoter region of the *phoPQ* operon was amplified with primers 142 and 143 and fused by SOE PCR to gene *phoQ*

amplified with primers 144 and 145. The resulting construct was inserted to plasmid pBAV-1K-T5-GFP[38] PCR linearized with primers 146 and 147 to create the complementation vector. The vector was transformed to strain Δ*phoQ* by electroporation and selected on MH agar containing 90 μg/mL kanamycin (Sigma).

Antibiotic susceptibility testing. Colistin susceptibility of all strains was determined using the Etest method. Briefly, the inoculum was prepared from colonies grown on a 5% sheep blood agar plate (Remel, Lenexa, KS) for 18 hours. Several colonies were suspended in 0.9% sterile saline (Remel) and adjusted to a concentration equivalent to a 0.5 McFarland turbidity standard. The suspension was used to streak a 100mm diameter MH agar plate and the Etest strip (bioMérieux, Marcy-l'Étoile, France) was placed. The plate was incubated at 35°C for 20 hours and the minimum inhibitory concentration (MIC) was read where inhibition of growth intersected the Etest strip. Small colonies that grew within the zone of inhibition were included in the MIC determination. Etest analyses of samples from mouse infections were plated directly from peritoneal lavage samples without subculturing. Population analysis profiles were performed by growing bacteria to mid-log phase, and then plating on MH agar containing various concentrations of colistin. Percentage colistin resistance was calculated as the number of bacteria that grew on 100μg/mL colistin divided by the number of bacteria that grew on MH alone.

Mice. Wild-type C57BL/6J mice were purchased from Jackson Laboratories (Bar, Harbor, ME) and used at age 8-10 weeks, all experiments using age- and sex-matched mice. Triple knockout (TKO) mice deficient in the gp91 component of the NADPH oxidase, lysozyme, and CRAMP, as well as double knockout mice lacking two of the indicated antimicrobials, were derived by crossing *cybb*$^{-/-}$ (gp91; from Jackson Laboratories), *lysM*$^{-/-}$ (lysozyme; generously provided by Dr. Daniel Portnoy, UC Berkeley), and *cnlp*$^{-/-}$ (CRAMP; Jackson Laboratories) mice. TKO mice were

investigated for health defects by histology and bacterial culture of various organs, with no overt health differences observed in uninfected TKO mice when compared to wild-type. Mice were housed under specific-pathogen free conditions in filter-top cages at Yerkes National Primate Center, Emory University, and provided food and water ad libitum. All experimental procedures were approved by the Emory University Institutional Animal Care and Use Committee. Sample size, reported in figure legends, was determined by allowing for significance by Mann-Whitney test ($n \geq 4$) while minimizing number of animals used, and thus 5 mice were used per group for the majority of experiments. No randomization or blinding was done in the animal studies.

Mouse infections. ~$5x10^7$ CFU were administered per mouse for infections to quantify bacterial load; ~$2x10^8$ CFU were administered for survival experiments. Bacterial inocula were suspended in phosphate buffered saline (PBS) and 100uL was inoculated intraperitoneally (i.p.) to each mouse. Colistin methanesulfonate was injected i.p. in 100uL PBS at a dosage of 10mg/kg/dose. Mice were monitored by weight, and were sacrificed if found to be below 80% starting weight, as mandated by IACUC protocol. Mice were sacrificed and liver, spleen and peritoneal lavage samples were collected into sterile PBS. Solid organ samples were homogenized using a tissue-tearor (BioSpec, Bartlesville, OK), and then all samples were plated for CFU and % colistin resistance.

Macrophage depletion. Macrophages were depleted from mice using clodronate liposomes (clodronateliposomes.com, Haarlem, Netherlands). Mice were injected with 200µL liposomes i.p. 3 days prior to infection, and then injected again with 100µL liposomes i.v. 1 day prior to infection. Mice were infected i.p. for 8 hours before peritoneal lavage fluid was harvested and plated for CFU. Part of this sample was also used for flow cytometry to confirm macrophage depletion.

Macrophage infection. Macrophages were derived from the bone marrow of mice. Briefly, femurs from mice were removed and whole bone marrow was flushed out. The bone marrow cells were grown in media containing Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS) and macrophage colony-stimulating factor (M-CSF) which induces the differentiation and growth of macrophages. After confluent layers of macrophages were derived, cells were plated into 24 well plates at $3x10^5$ cells per well. Bacteria were added to the wells at $3x10^6$ CFU per well for a multiplicity of infection (MOI) of 10:1. Plates were centrifuged to synchronize the infection. After 30 minutes, the macrophages were washed and 100μg/mL of gentamicin was added to the media to remove and prevent growth of extracellular bacteria. At 1, 2, and 4 hours post infection, macrophages were incubated with 1% saponin in PBS for 2 minutes to lyse open cells and remove bacteria. Samples were then plated for CFU and % colistin resistance calculated. To prevent internalization of bacteria, some wells were pretreated with 1 μg/mL of cytochalasin D for 30 minutes before addition of bacteria.

Flow cytometry. Peritoneal lavage fluid was stained with F4/80-PE/Cy7 (BM8) (Biolegend, San Diego, CA) and CD11b-APC/A700 (M1I70) (eBioscience, San Diego, CA) antibodies for 35 minutes. Red blood cells were lysed with RBC lysis buffer (Becton Dickinson, Franklin Lakes, NJ) for 5 minutes. Cells were fixed with 1% paraformaldehyde and analyzed on an LSRII flow cytometer (BD). Macrophages were defined as F4/80+CD11b+ cells.

DNA and RNA Isolation. An overnight liquid culture of R/S grown at 37°C in MH broth was back diluted in triplicate to either fresh MH broth or MH broth containing 100μg/mL colistin to enrich for susceptible or colistin resistant bacteria, respectively. Cultures were grown to exponential phase at 37°C and harvested for DNA and RNA isolation. CFU were calculated as above. DNA was isolated using the DNEasy Blood and Tissue Kit (Qiagen) following the Gram

negative bacteria protocol with RNase treatment. RNA was isolated using a modified phase extraction method[39] with initial incubation in TriReagent (Zymo) followed by phase separation with chloroform. RNA was precipitated from the aqueous phase with isopropanol and 1.2M NaCl at 4°C and further purified with the Directzol RNA Kit (Zymo) following the recommended DNase treatment step.

DNA and RNA sequencing. Sample integrities were verified with the Agilent 2100 Bioanalyzer (Agilent). DNA libraries were prepared using the NexteraXT DNA kit (Illumina). For RNA libraries, samples were first depleted of ribosomal RNAs using the Ribo-Zero rRNA Removal Kit (Illumina) and libraries prepared using the EpiCentre ScriptSeq Complete (Bacteria) Low Input kit (Illumina). Next generation short sequence reads were generated with the Illumina HiSeq 1000 platform at the Yerkes National Primate Research Center Nonhuman Primate Genomics Core (http://www.yerkes.emory.edu/nhp_genomics_core/). Long sequence reads were generated with the PacBio II platform using the P5-C3 chemistry at the Duke University Sequencing and Genomic Technologies Shared Resource.

***De novo* genome assembly and sequence analysis.** A hybrid de novo assembly was performed using both Illumina and PacBio data using Celera Assembler version 8.2[40]. The sequence data resolved into two contigs, one representing the chromosome and the other representing the plasmid. Quality of the assembly was confirmed by analysis using the ALE tool[41]. The assembly was automatically annotated using the NCBI prokaryotic annotation pipeline. Illumina whole shotgun sequences of the samples enriched for colistin resistance (COL) and colistin susceptibility (MH) were aligned against the assembled genome using bwa-0.7.12[42] and visualized the samtools-1.2 mpileup function[43]. Single nucleotide polymorphisms between the assembled genome and

short sequence reads were manually analyzed to determine sequence conservation between COL and MH samples.

RNAseq analysis. Single end Illumina libraries from reverse-transcribed RNA were mapped against the Enterobacter de novo assembled reference using Bowtie2[44]. Differential gene expression between the three colistin-treated strains and controls was quantified by the cufflinks/cuffdiff tools in CufflinksVersion 2.2.1[45,46]. Sequences of differentially expressed genes with significant q-values were analyzed with Blast2Go software version 3.1.3 to identify the *Escherichia coli* gene ortholog and putative function[47].

Quantitative Reverse Transcriptase PCR (qRT-PCR). RNA was harvested as above. One-step qRT-PCR was performed using the Power SYBR Green RNA-to-C_t kit (Applied Biosystems) with primers **(Table S4)** on a StepOnePlus Real-time PCR System (Applied Biosystems) according to the manufacturer's instructions. *rpoD* was used as the internal control gene[48]. Relative expression was calculated as $2^{-(\Delta Ct)}$.[49]

Isolation and analysis of ^{32}P Lipid A species. *E. cloacae* strains were grown overnight in MH broth, diluted 1:400 in fresh MH broth containing appropriate selective antibiotics. For induction of resistant phenotype, 100 μg/ml colistin (Sigma) was used. *Escherichia coli* W3110 and WD101 strains were grown in LB broth overnight followed by a 1:100 dilution in fresh LB medium. After dilutions, cells were immediately labeled with 2.5 μCi/ml of inorganic ^{32}P-phosphate (Perkin Elmer) and harvested at A_{600} 0.5 (*E. cloacae*) or A_{600} 1.0 (*E. coli*). Lipid A extraction, separation and visualization was performed as previously described[50]. Briefly, lipid A extraction was carried out by mild acidic hydrolysis and spotted onto silica TLC plate (10,000 cpm/lane). Labeled lipid A species were separated using a solvent mixture of chloroform, pyridine, 88% formic acid and water (50:50:16:5). TLC plate was exposed to a phosphoimager screen and visualized by

phosphoimaging analysis (Bio-Rad PMI). The analyzed images were cropped to aid in data analysis; the full unaltered images are available in Supplementary Figure 24.

Statistics. Statistical analyses were performed using Prism 5 (Graphpad Software). Significance of mouse experiments was determined with the Mann Whitney test, as not all data were normally distibuted, while all *in vitro* experiments were analyzed using the two-tailed student's t-test (on data with normal distribution). All experiments were repeated at least 2-3 times (and up to 10 times). All replicates shown are biological replicates.

Accession. DNA and RNA sequencing data were deposited at NCBI under Bioproject number PRJNA263343 as BioSamples SAMN03099688, SAMN04538424, and SAMN04538425.

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Supplementary Figures

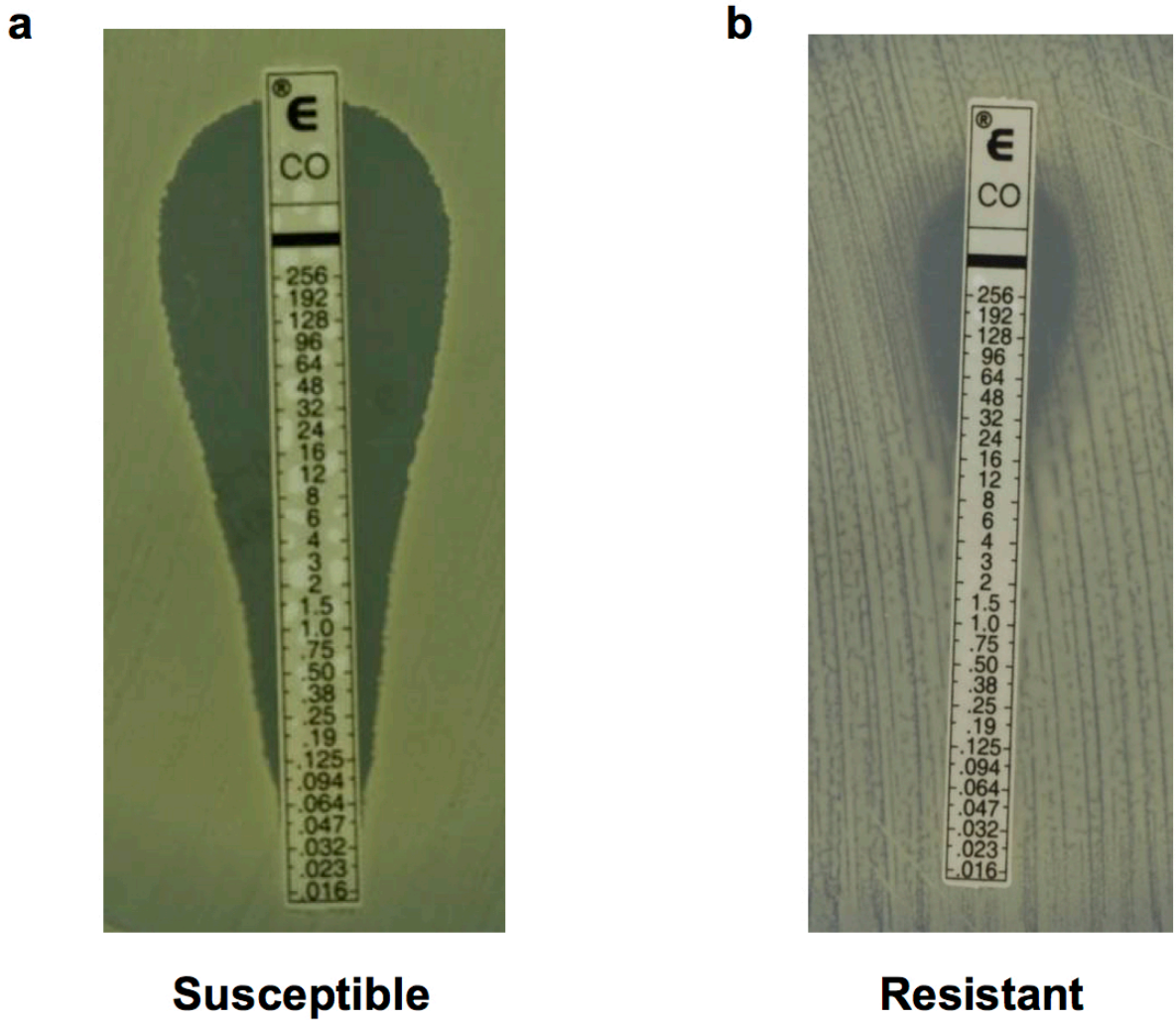

Figure S1

Figure S1. 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.

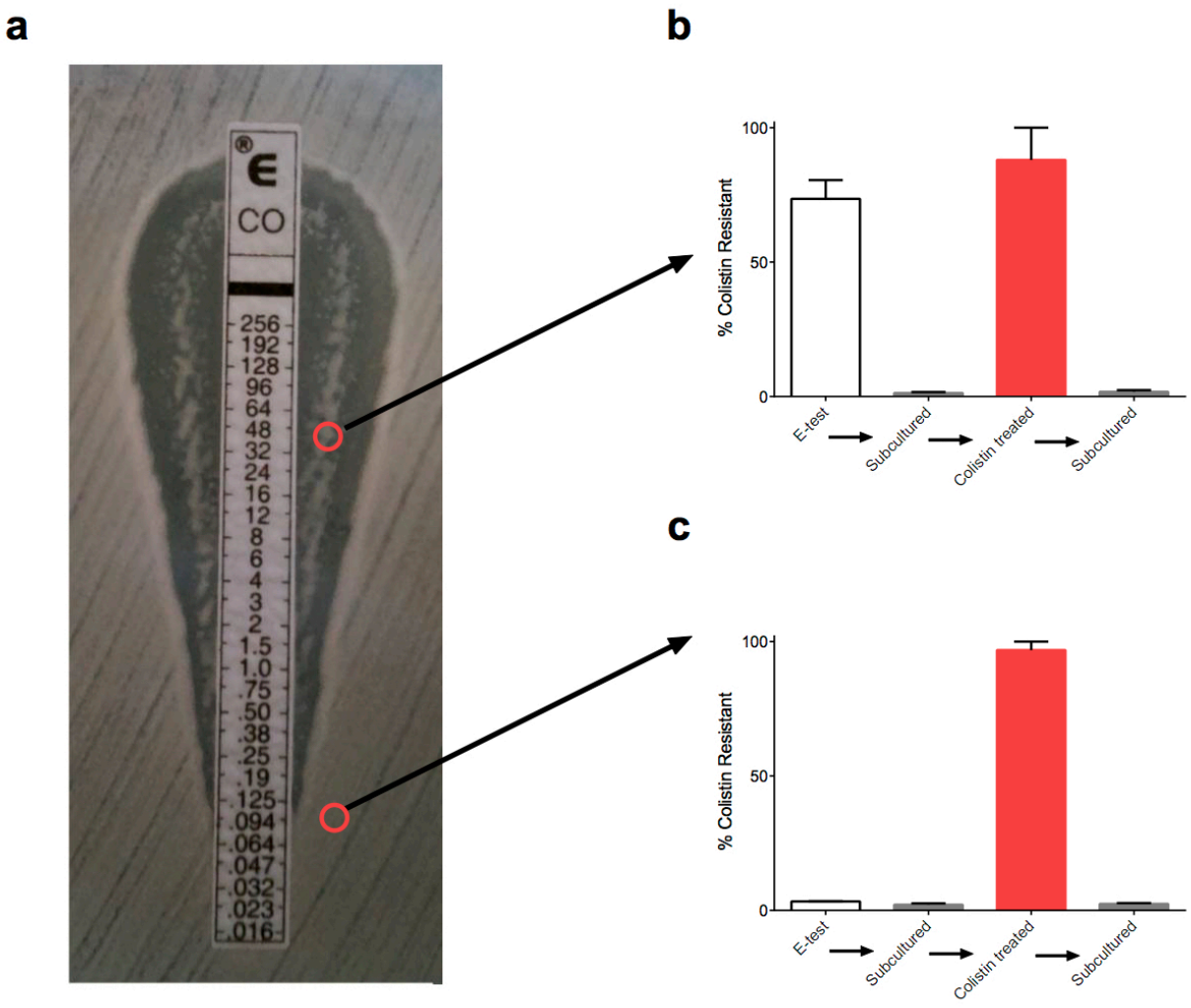

Figure S2

Figure S2. 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,.

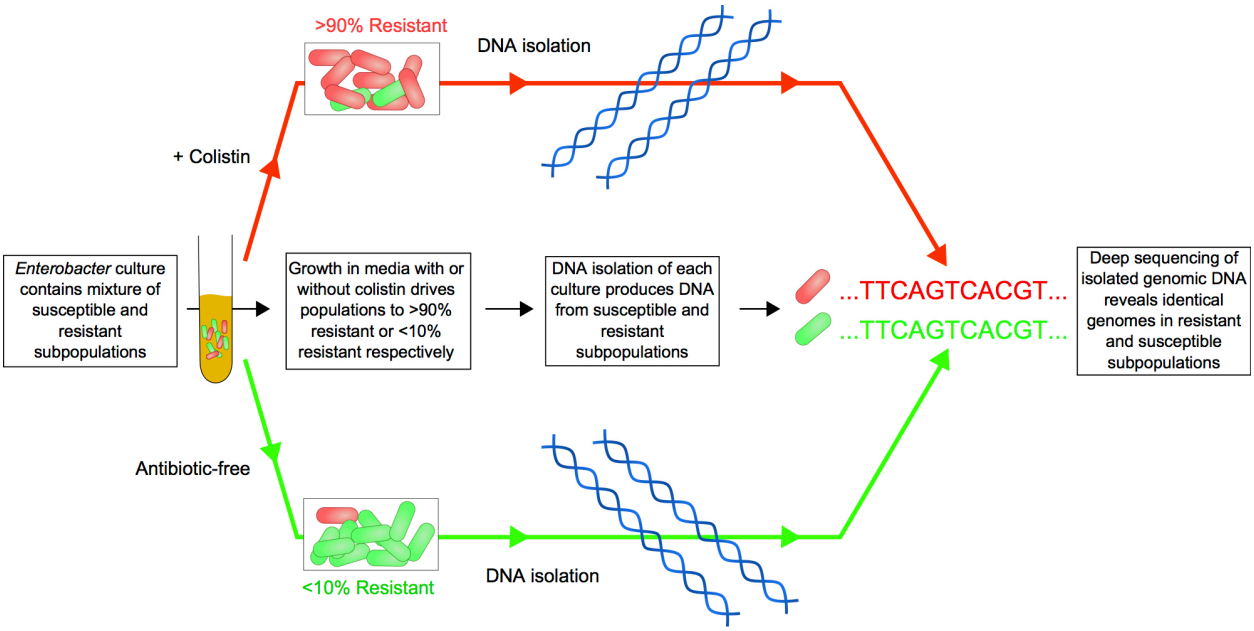

Figure S3

Figure S3. 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).

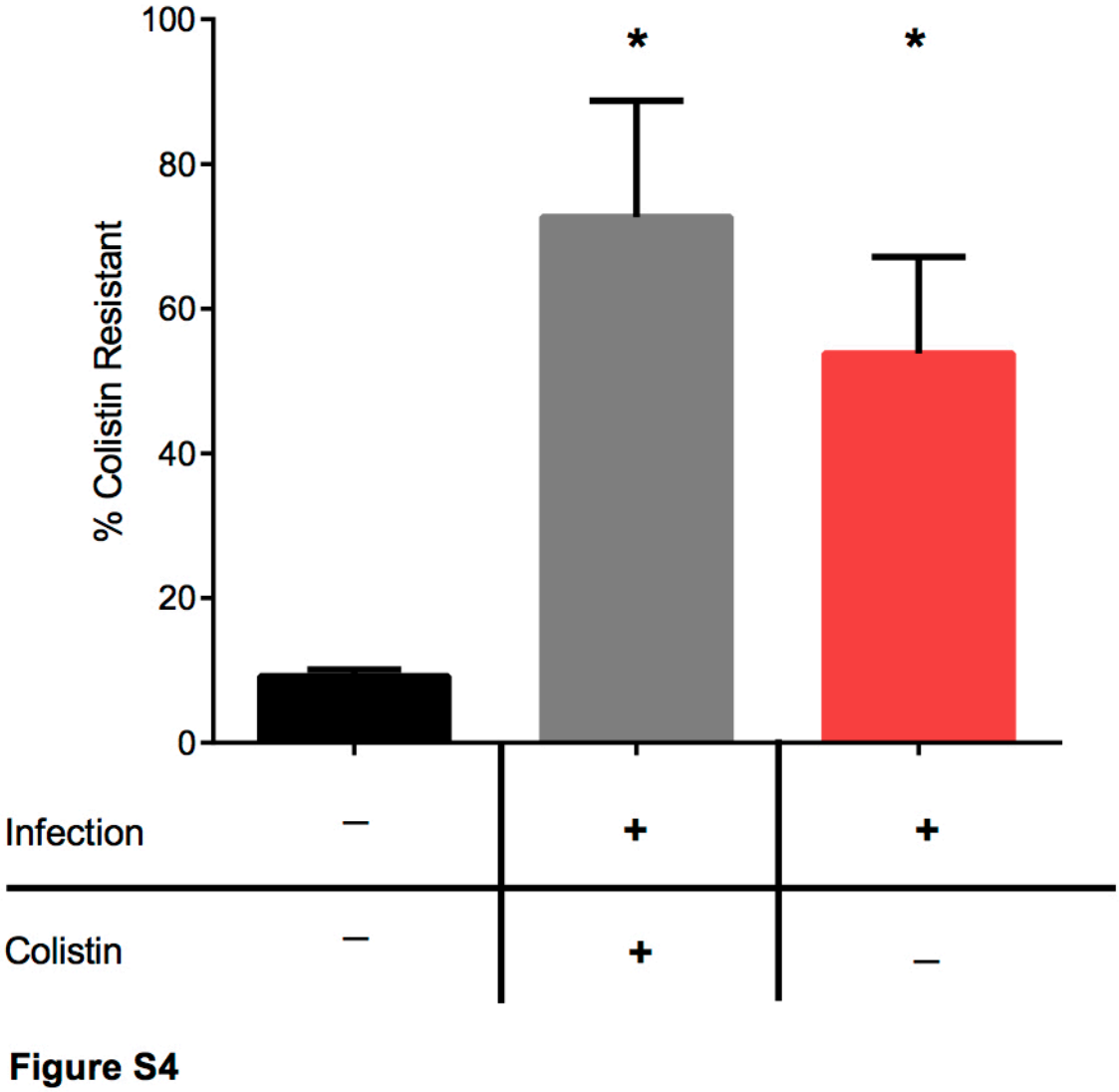

Figure S4

Figure S4. 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, * $p < 0.05$).

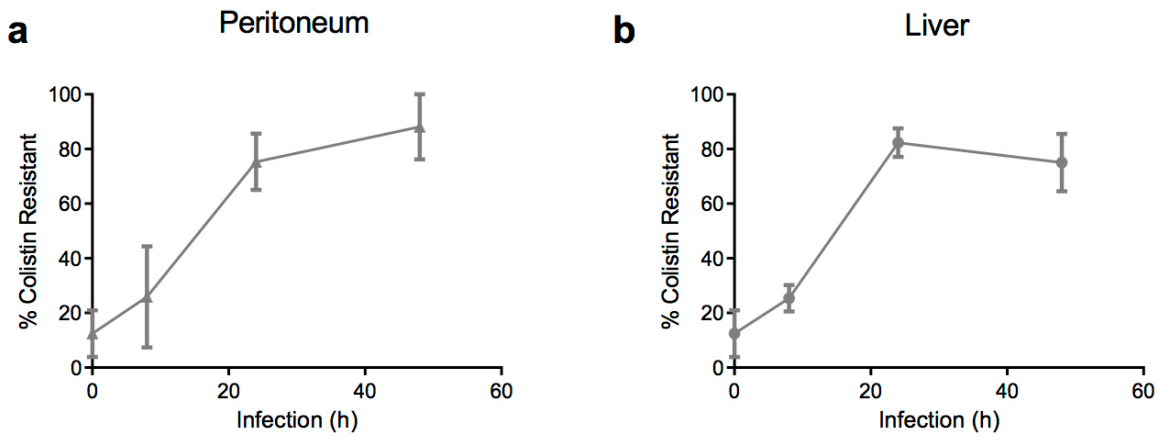

Figure S5

Figure S5. 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.

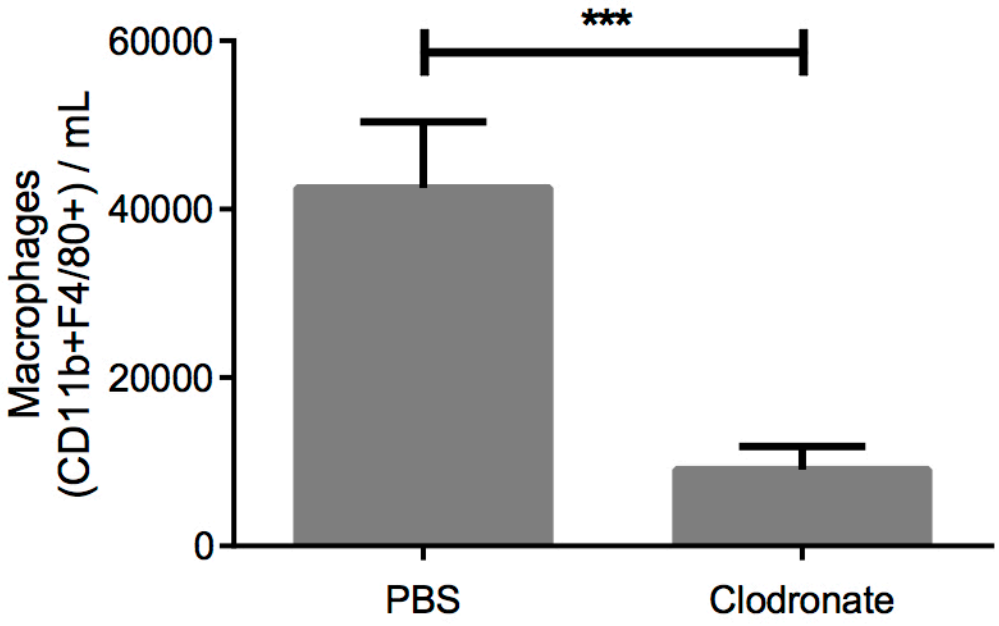

Figure S6

Figure S6. 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, ***, $p < 0.001$.)

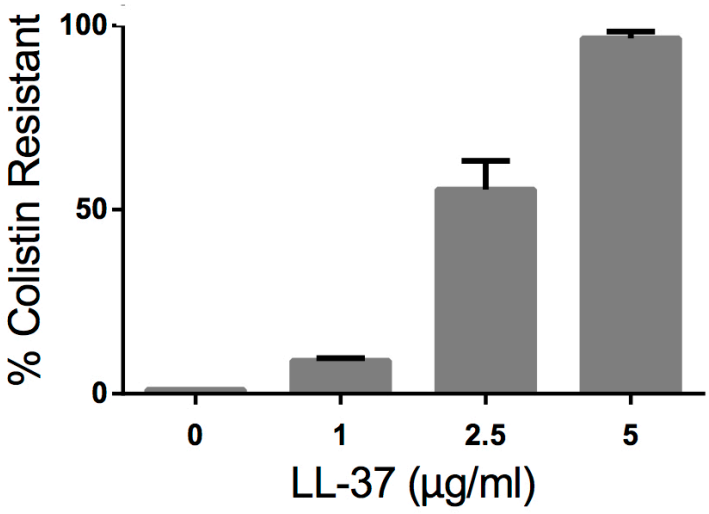

Figure S7. The human antimicrobial peptide LL-37 leads to an increase in frequency of the colistin resistant subpopulation. Strain R/S was treated with the indicated amounts of human LL-37 for 5 hours. Samples were plated to quantify the numbers of total and colistin-resistant bacteria and % colistin resistance was calculated (n = 3). Error bars represent s.e.m.

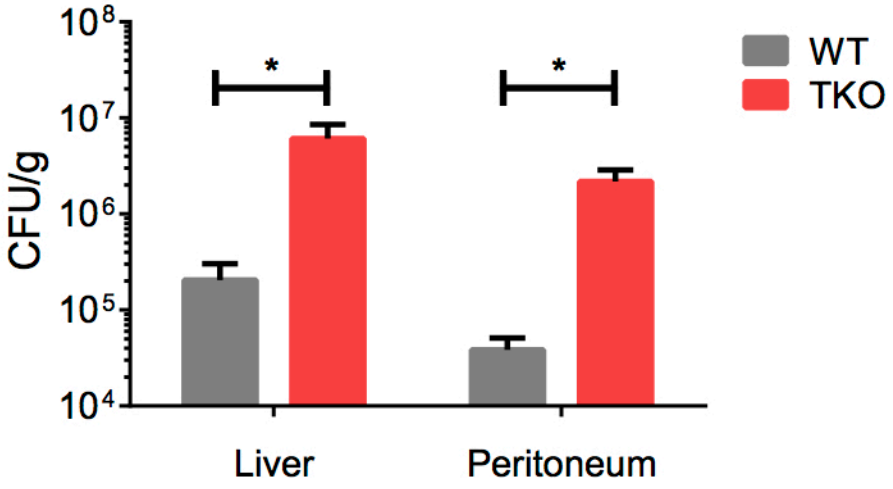

Figure S8

Figure S8. 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, * $p < 0.05$).

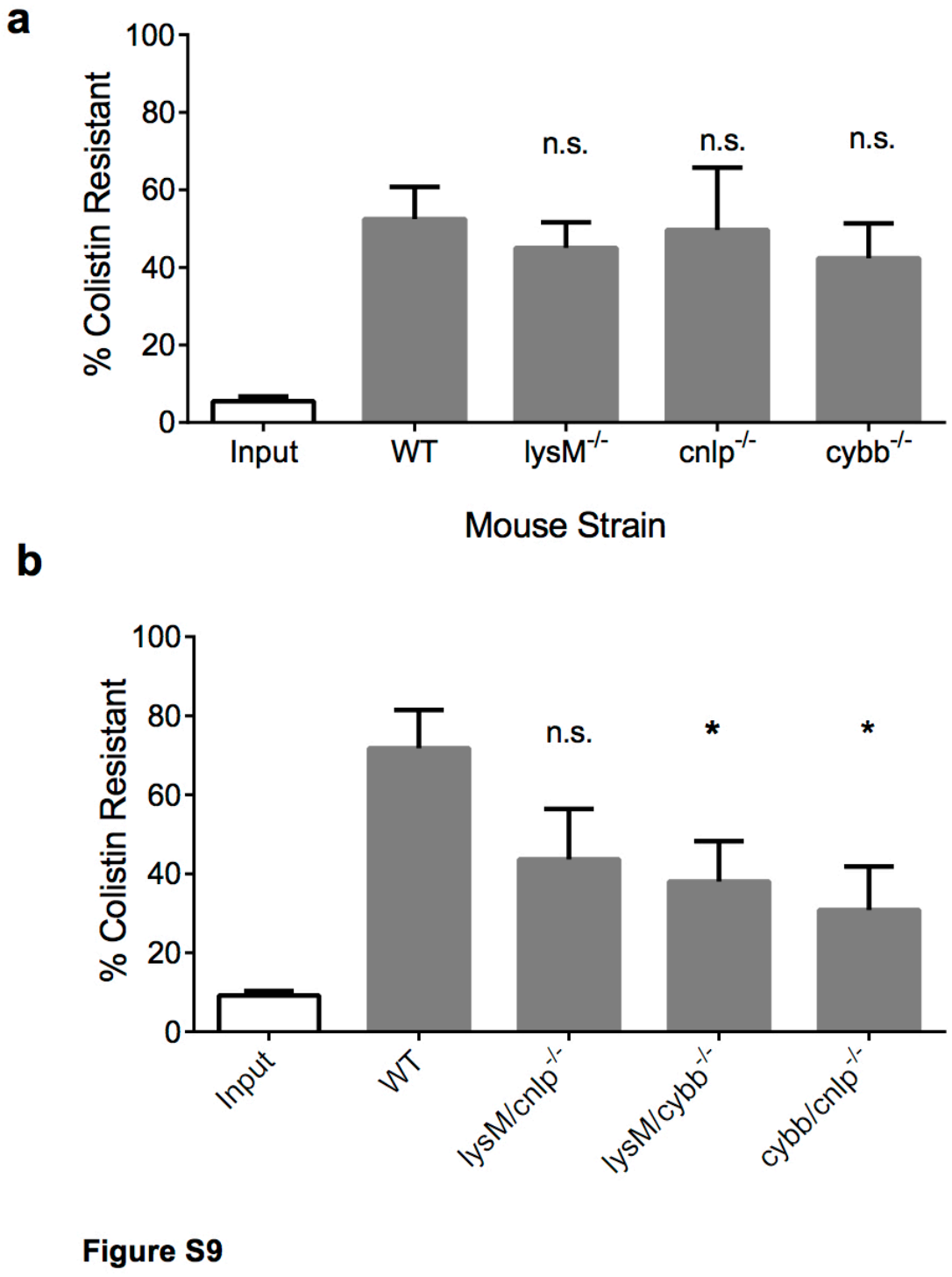

Figure S9

Figure S9. 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).

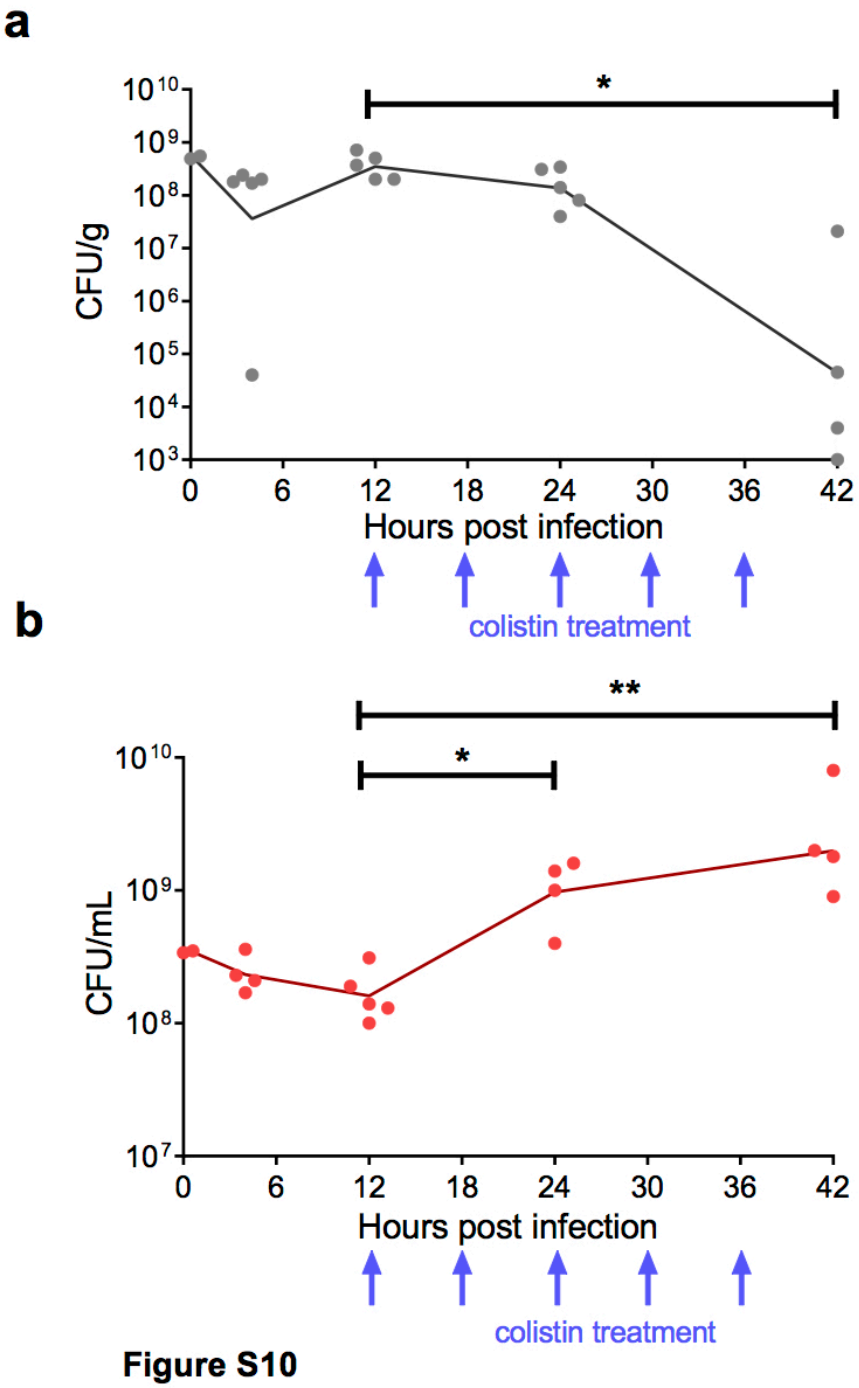

Figure S10. *In vivo* growth and expansion of R/S during colistin treatment of mice. Wild-type mice were infected with a lethal dose of (**a**) a susceptible strain (n = 4 to 6) or (**b**) R/S (n = 4 to 6) and then given doses of colistin every six hours starting at 12 hours post infection. Mice were

sacrificed to determine peritoneal CFU at 0, 6, 24 and 42 hours. Error bars represent s.e.m., lines represent median. (Mann-Whitney test, * $p < 0.05$, ** $p < 0.01$).

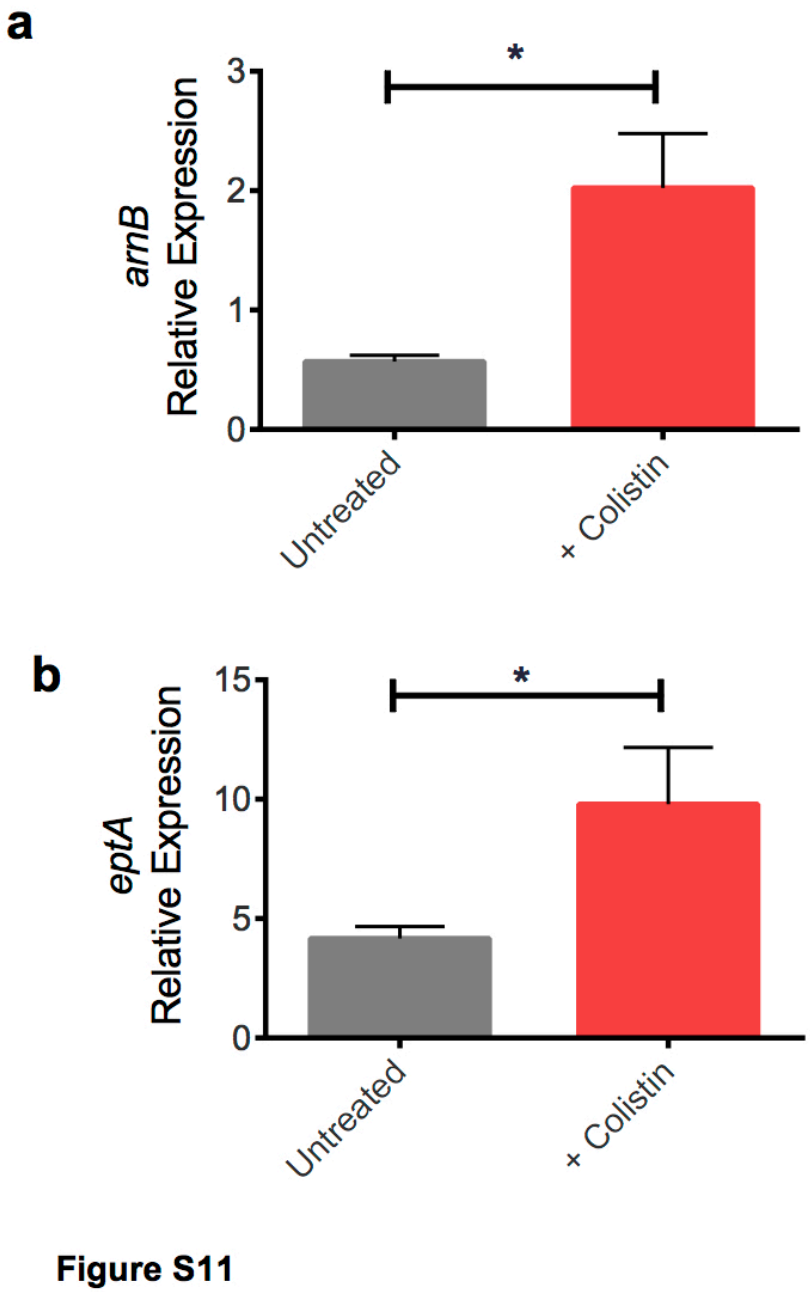

Figure S11

Figure S11. 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 Figure S3 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, * $p < 0.05$).

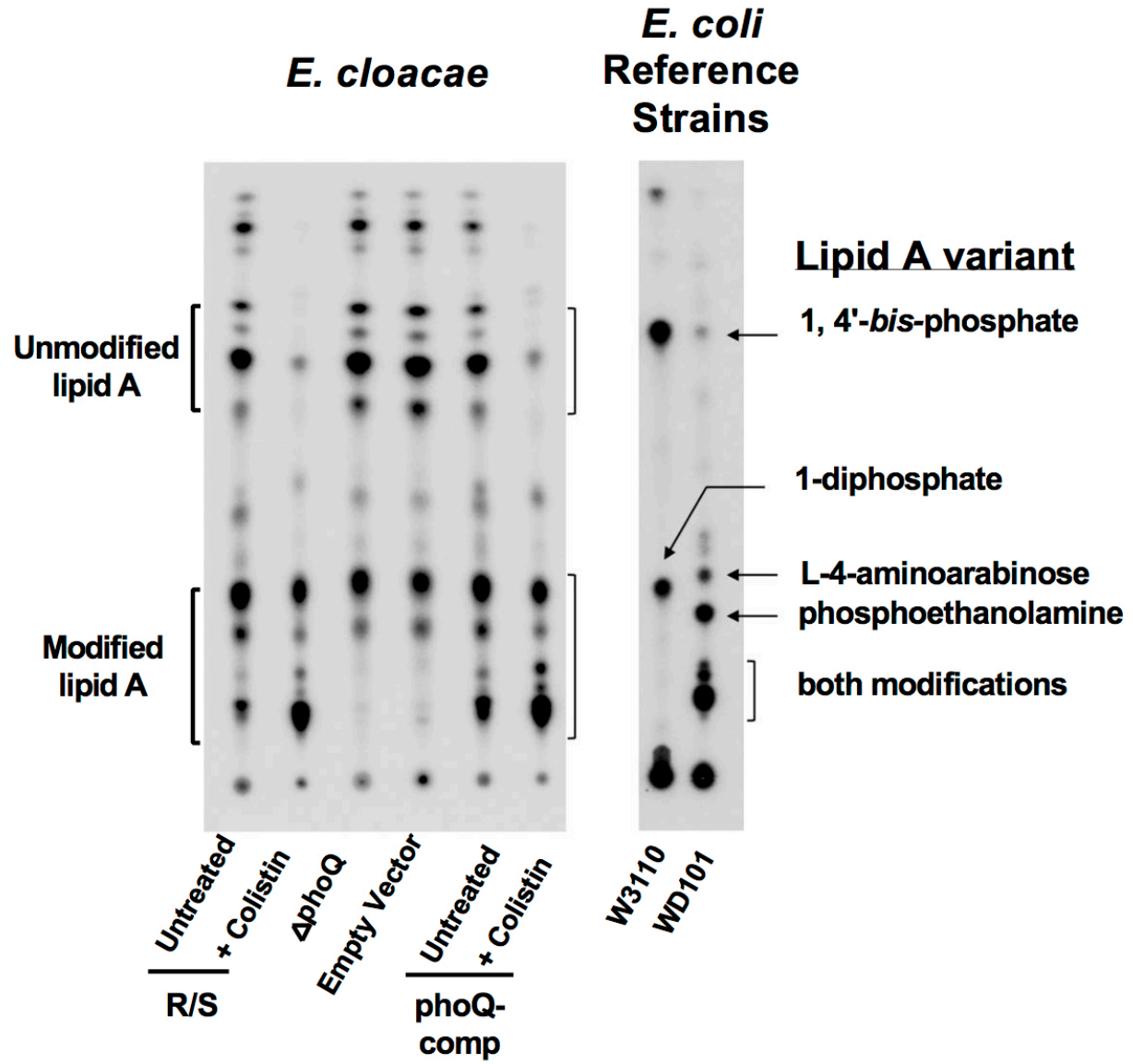

Figure S12

Figure S12. 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 Figure S3 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).

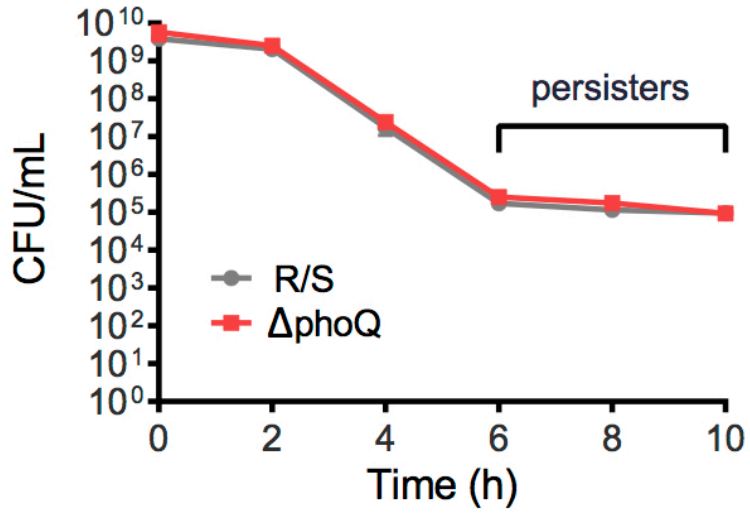

Figure S13

Figure S13. 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.

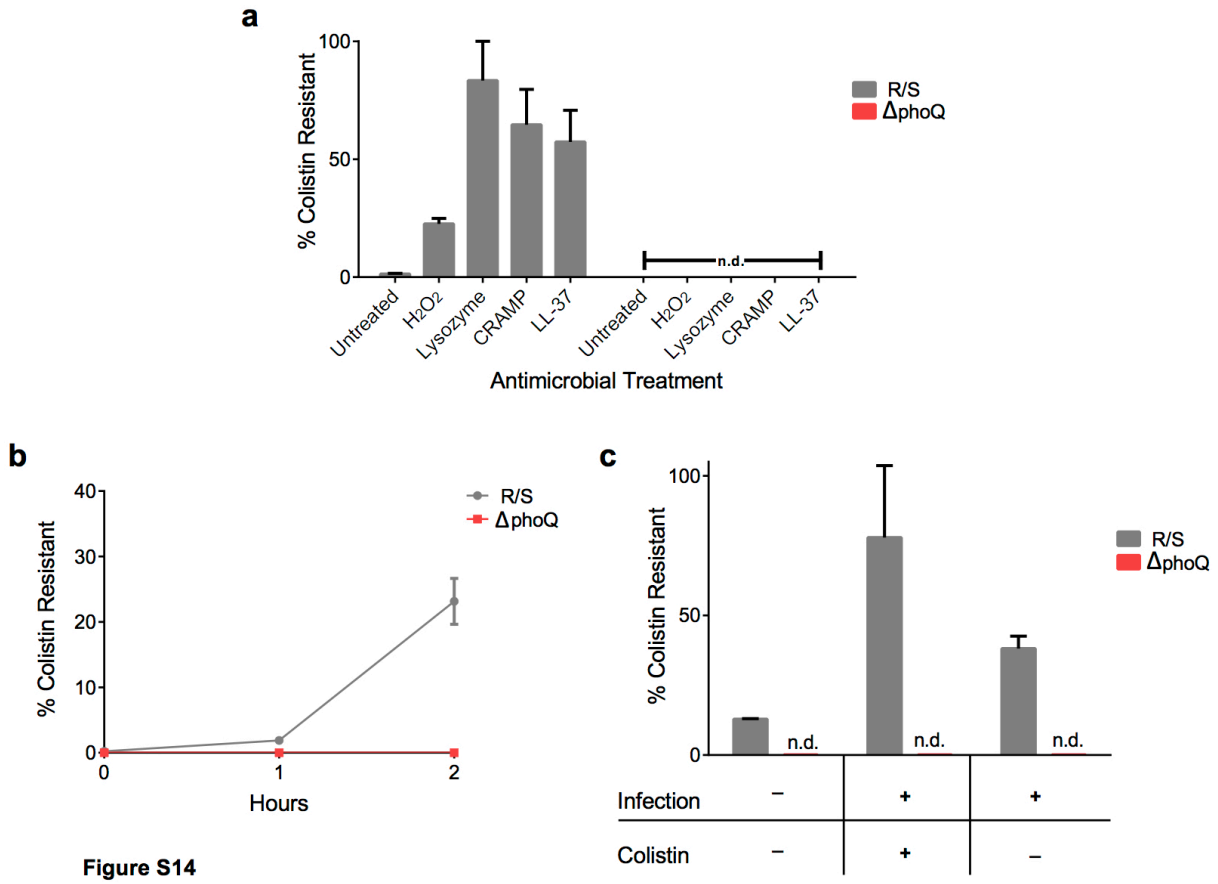

Figure S14

Figure S14. 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.

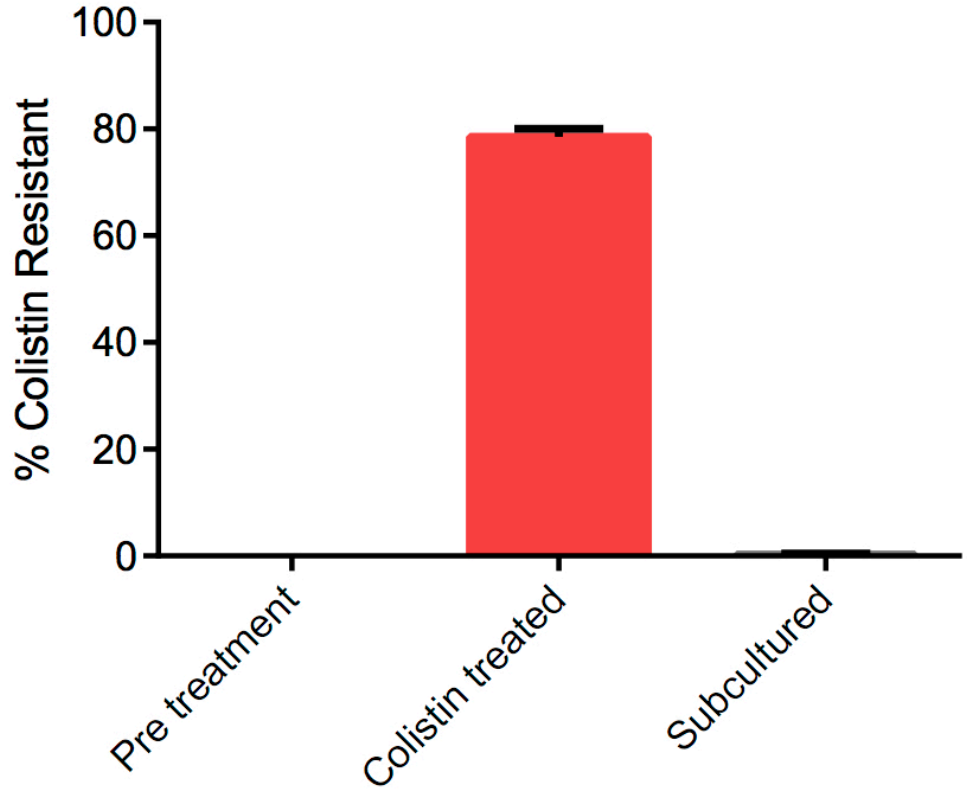

Figure S15

Figure S15. 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.

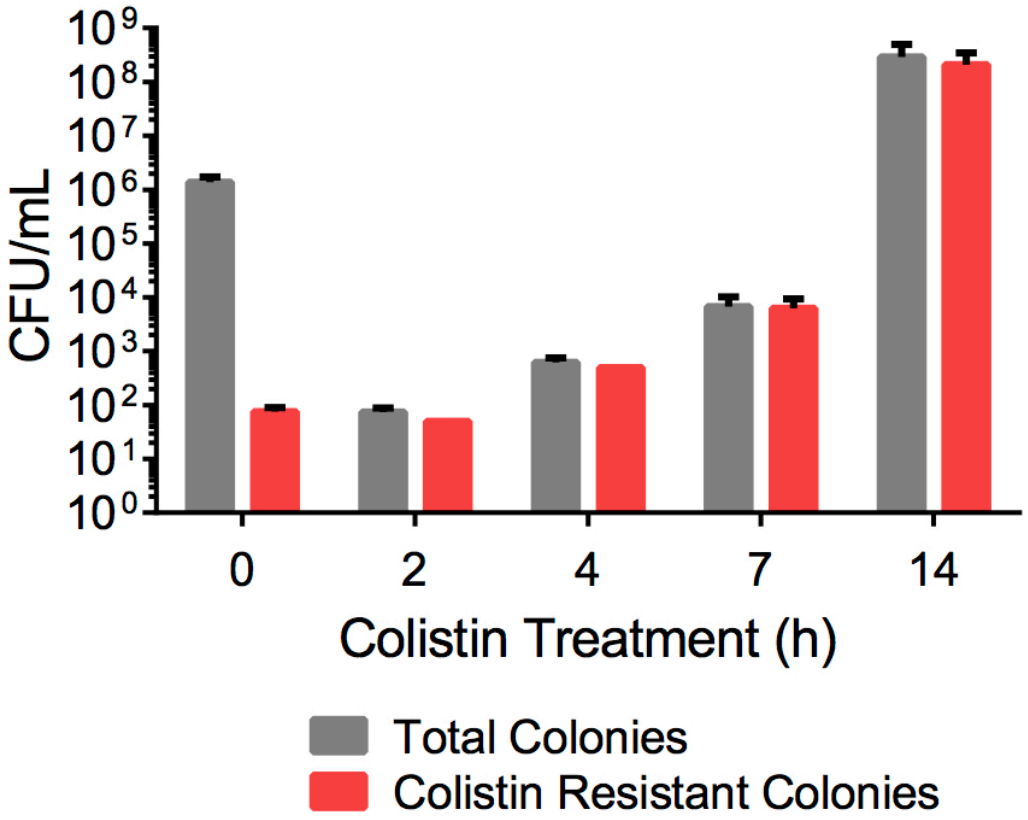

Figure S16

Figure S16. 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.

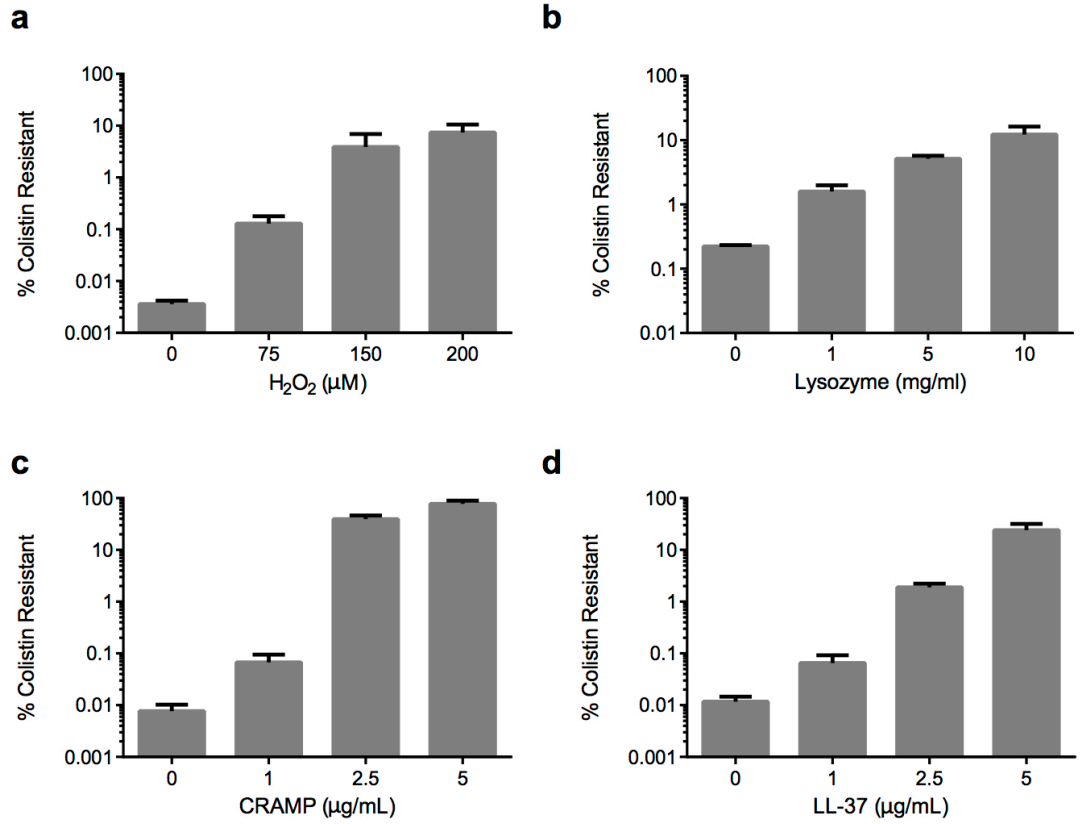

Figure S17

Figure S17. 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.

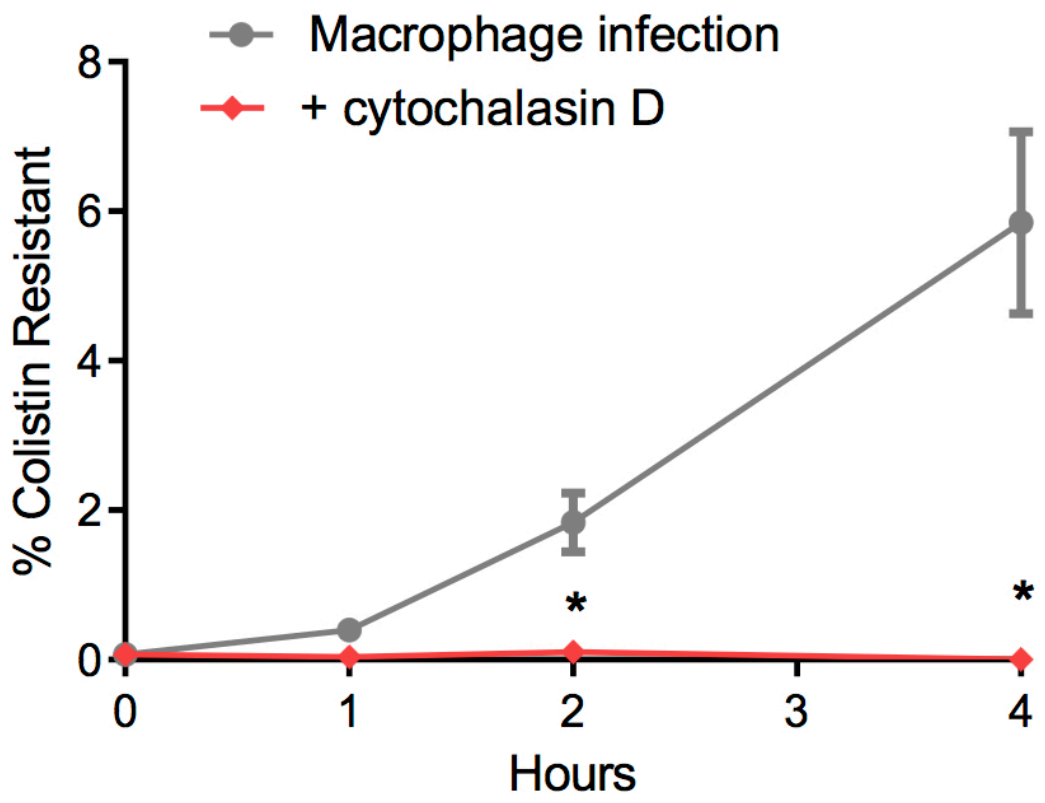

Figure S18

Figure S18. 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, * $p<0.05$).

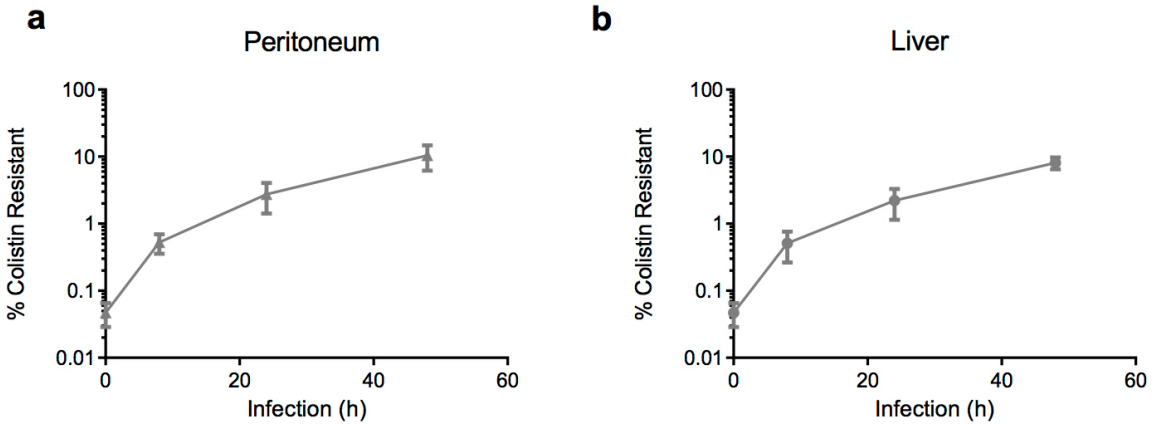

Figure S19

Figure S19. 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.

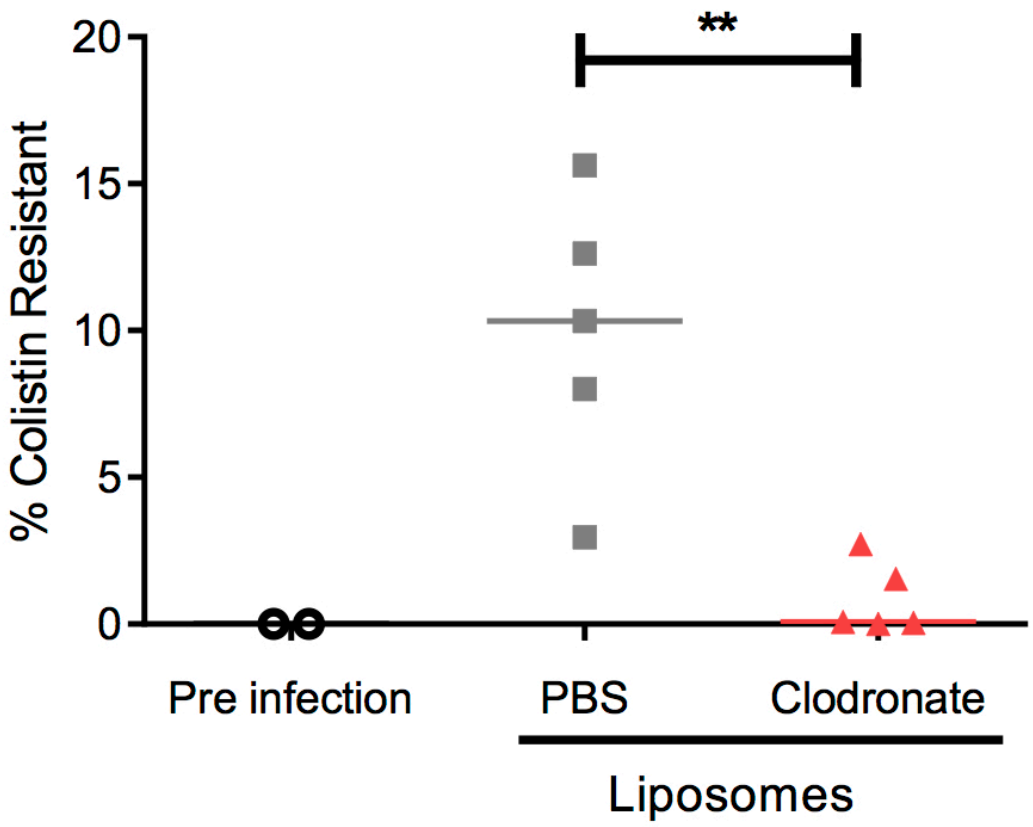

Figure S20

Figure S20. 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, ** $p < 0.01$).

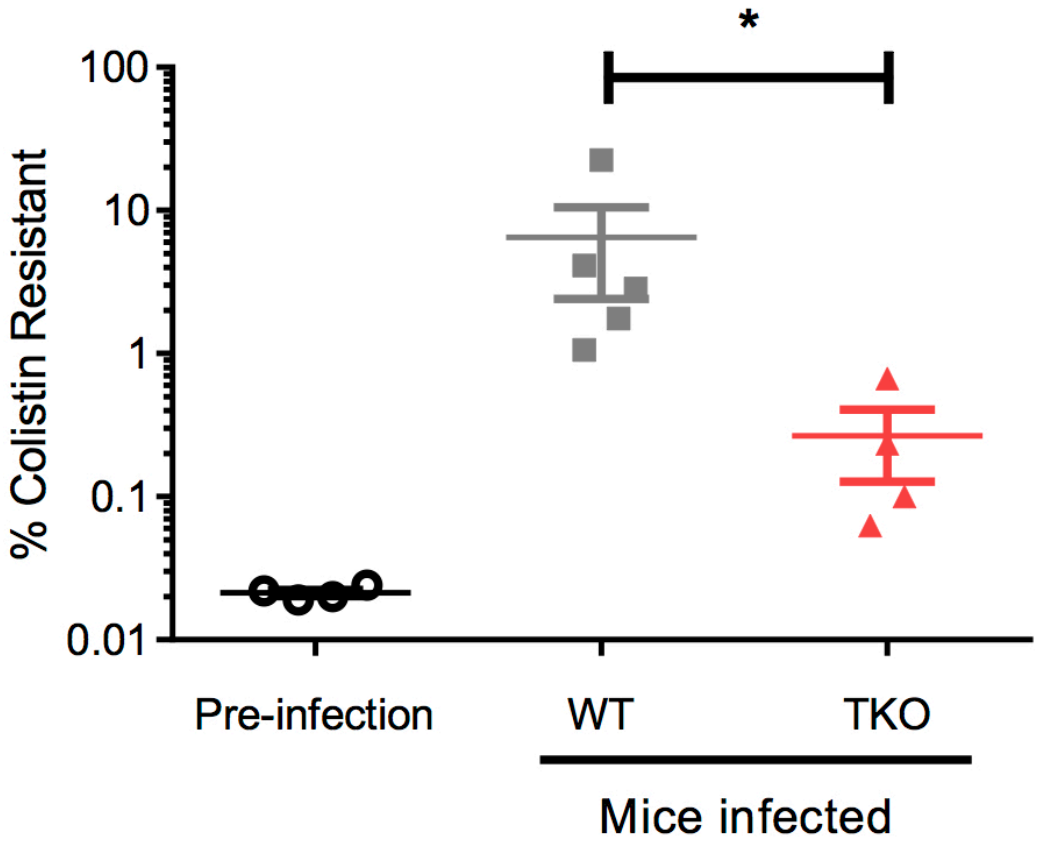

Figure S21

Figure S21. 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, * $p < 0.05$).

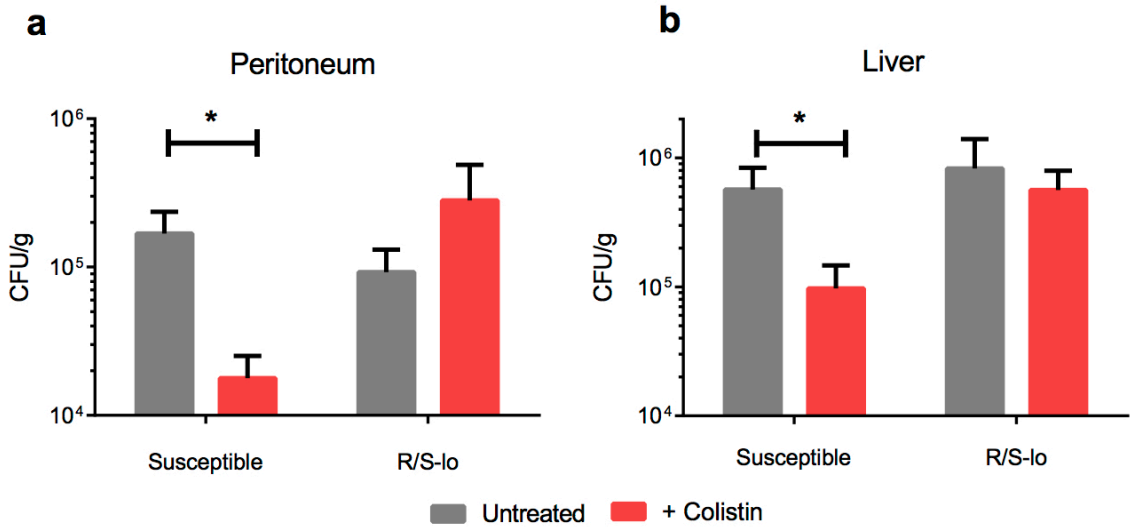

Figure S22

Figure S22. 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, * $p < 0.05$.

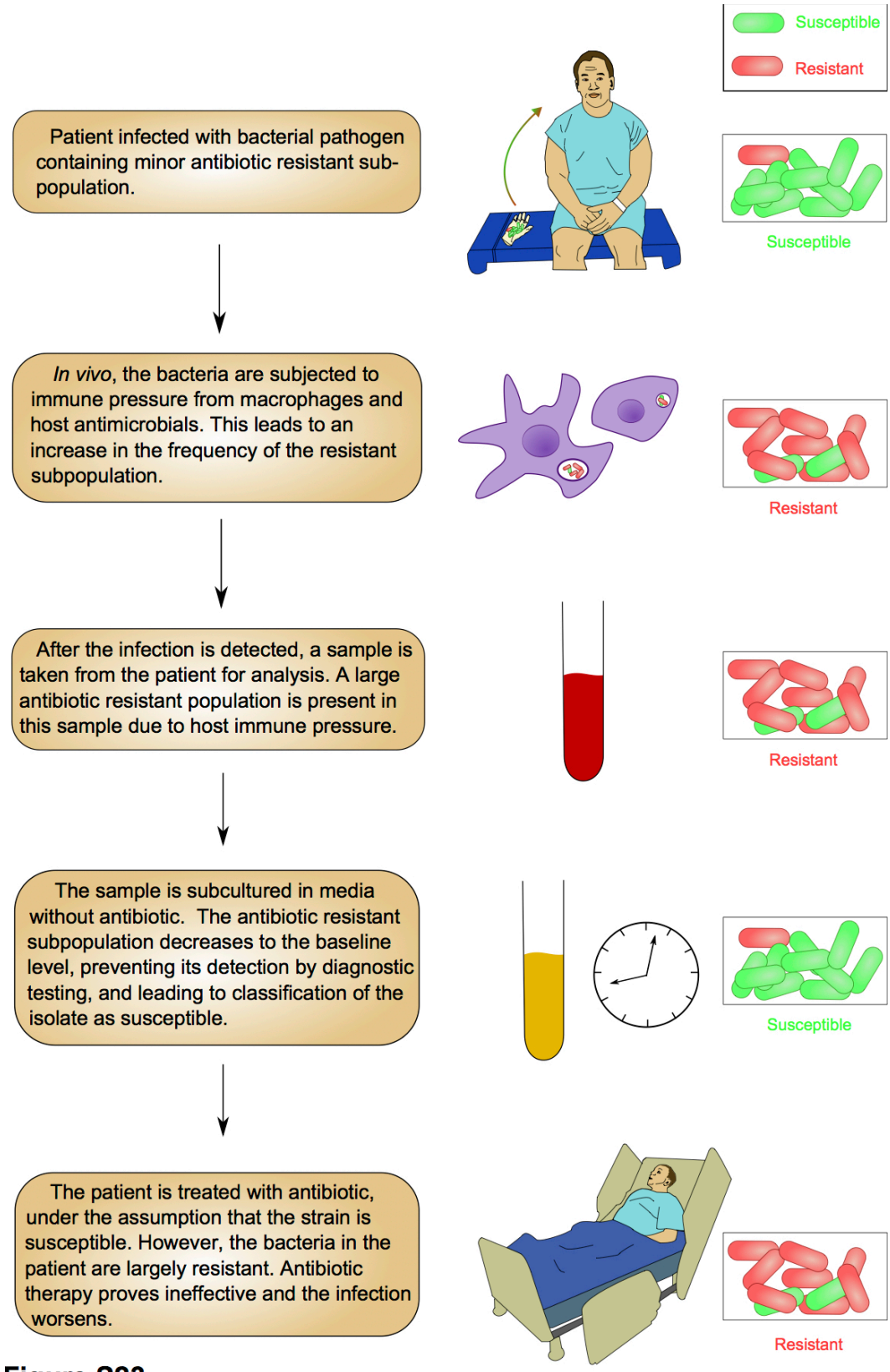
Susceptible
Resistant
Patient infected with bacterial pathogen containing minor antibiotic resistant sub-population.
Susceptible
In vivo, the bacteria are subjected to immune pressure from macrophages and host antimicrobials. This leads to an increase in the frequency of the resistant subpopulation.
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

Figure S23

Figure S23. 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.

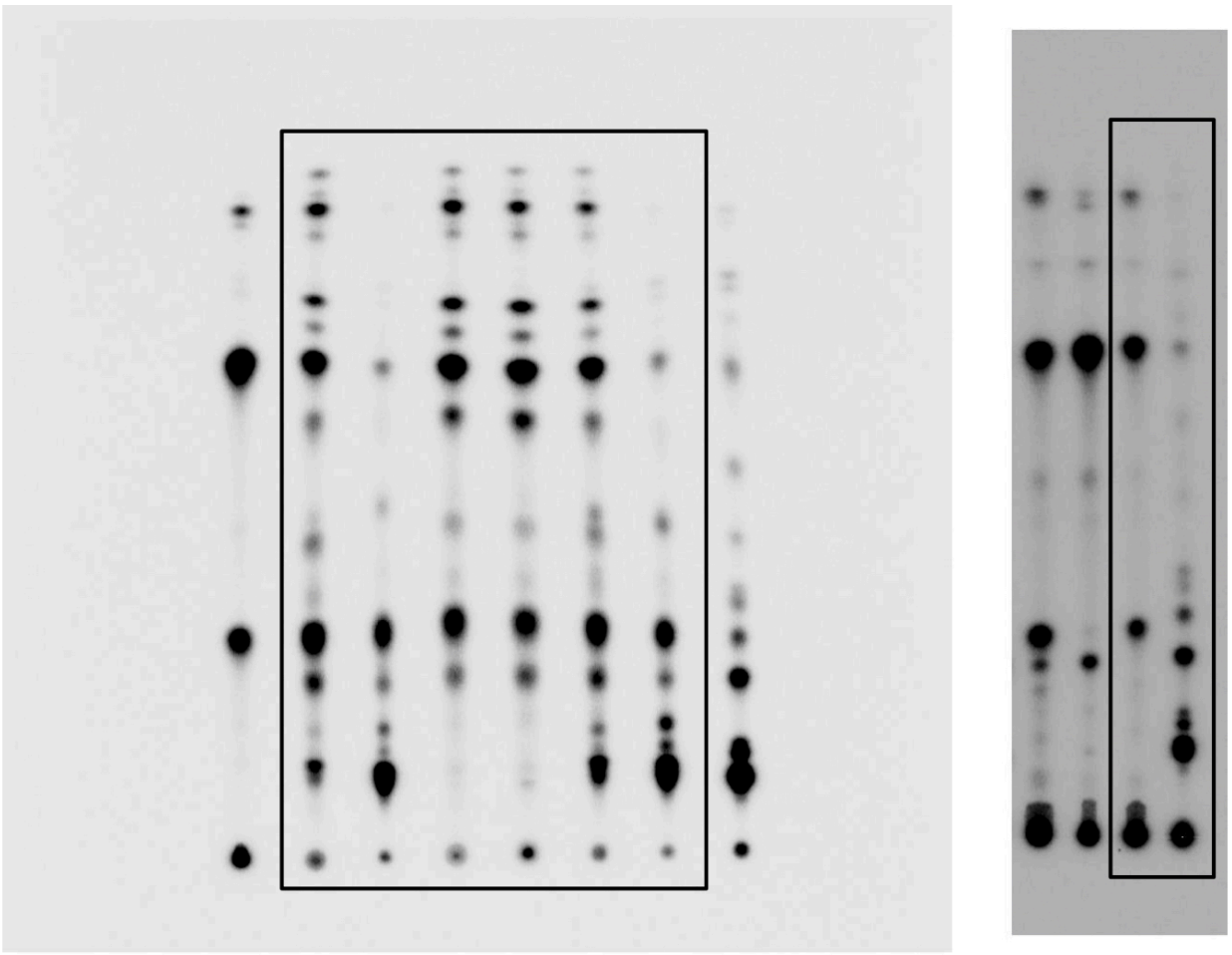

Figure S24

Figure S24. Raw image files of lipid A thin layer chromatography.

Supplementary Tables

Supplementary Table 1. Gene expression of untreated and colistin treated strain R/S by RNAseq.

*File not included due to excessive length. Contains complete RNAseq gene expression data for entire chromosome and plasmid of *E. cloacae* strain R/S.

Supplementary Table 2. Genes Differentially Expressed in the Colistin Treated Subpopulation of strain R/S by RNAseq.

Locus	Gene Homolog	Predicted Function	Log_2 Expression Fold Change Induced	q value
NF29_17760	*None*	---NA---	6.89422	<0.001
NF29_17775	*None*	---NA---	5.53341	<0.001
NF29_17780	*None*	---NA---	4.39284	<0.001
NF29_13050	*wcaH*	gdp-mannose mannosyl hydrolase	4.14589	<0.001
NF29_13045	*wcaG*	nucleotide di-p-sugar epimerase or dehydratase	4.06472	<0.001
NF29_00670	*None*	---NA---	3.87778	<0.001
NF29_13065	*cpsG*	phosphoglucomutases and phosphomannomutases	3.80107	<0.001
NF29_13005	*wzc*	tyrosine-protein kinase	3.6544	<0.001
NF29_13055	*wcaI*	colanic biosynthesis glycosyl transferase	3.52877	<0.001
NF29_17785	*acrB*	acridine efflux pump	3.4121	<0.001
NF29_13060	*cpsB*	mannose-1-phosphate guanyltransferase	3.40668	<0.001
NF29_13040	*gmd*	gdp-d-mannose dehydratase	3.36701	<0.001
NF29_09375	*pgaB*	poly-beta-1,6-N-acetyl-D-glucosamine N-deacetylase	3.33118	<0.001
NF29_12995	*wza*	polysaccharide export protein	3.2636	<0.001
NF29_13010	*wcaA*	colanic acid biosynthesis glycosyltransferase	3.26267	0.047
NF29_16365	*ydeI*	hydrogen peroxide resistance protein	3.23897	<0.001
NF29_13000	*wzb*	probable protein-tyrosine-phosphatase	3.21338	<0.001
NF29_09380	*pgaA*	outer membrane protein	3.1711	<0.001
NF29_10190	*None*	---NA---	3.0716	<0.001
NF29_13080	*wcaK*	galactokinase (ec)	3.01598	<0.001
NF29_13035	*wcaF*	thiogalactoside acetyltransferase	2.95859	<0.001
NF29_13410	*None*	---NA---	2.91173	<0.001
NF29_12905	*fbaB*	fructose-bisphosphate aldolase class I	2.86936	<0.001
NF29_20480	None	---NA---	2.82854	<0.001
NF29_07135	*arnA*	10-formyltetrahydrofolate:l-methionyl-trna n-formyltransferase	2.72271	<0.001
NF29_13020	*wcaC*	glycosyl transferase	2.72053	0.0332956
NF29_13070	*wcaJ*	colanic acid biosynthsis udp-glucose lipid carrier transferase	2.70866	<0.001
NF29_09370	*pgaC*	polysaccharide metabolism	2.69565	<0.001
NF29_21650	*entC*	isochorismate hydroxymutase enterochelin biosynthesis	2.68738	<0.001
NF29_07130	*arnC*	sugar transferase	2.65771	0.0152405
NF29_01875	*mgtA*	mg2+ transport p-type 1	2.65585	<0.001

NF29_18075	*eptA*	lipid A phosphoethanolamine transferase	2.60527	<0.001
NF29_13075	*wzxC*	probable export protein	2.58226	<0.001
NF29_13405	*None*	---NA---	2.56454	<0.001
NF29_21645	*entE*	dihydroxybenzoate-amp ligase	2.55835	0.00312064
NF29_16360	*yghA*	2-deoxy-d-gluconate 3-dehydrogenase	2.55599	<0.001
NF29_07140	*arnD*	undecaprenyl phosphate-alpha-L-ara4FN deformylase	2.52871	0.0203026
NF29_04500	*ORF_o222*	hypothetical protein	2.47583	<0.001
NF29_05565	*ibpB*	heat shock protein	2.45974	<0.001
NF29_19495	*None*	---NA---	2.45174	<0.001
NF29_20485	*ybjG*	undecaprenyl pyrophosphate phosphatase	2.45085	<0.001
NF29_13420	*None*	---NA---	2.4438	<0.001
NF29_04495	*yjbG*	polysaccharide export OMA protein	2.44034	0.00436494
NF29_20990	*ybgS*	homeobox protein	2.41473	<0.001
NF29_07125	*arnB*	uridine 5'-(beta-1-threo-pentapyranosyl-4-ulose diphosphate) aminotransferase, PLP-dependent	2.41056	<0.001
NF29_13085	*wcaL*	colanic acid biosynthesis glycosyl transferase	2.38204	<0.001
NF29_16230	*ydgJ*	virulence factor	2.28545	<0.001
NF29_13030	*wcaE*	colanic acid biosynthesis glycosyl transferase	2.20957	<0.001
NF29_02655	*fieF*	transport system permease protein	2.19688	<0.001
NF29_15585	*yehX*	atp-binding component of a transport system	2.18695	<0.001
NF29_16270	*None*	---NA---	2.17386	<0.001
NF29_19375	*efeU*	ferrous iron transporter	2.14854	<0.001
NF29_11685	*tktB*	transketolase 2 isozyme	2.14843	<0.001
NF29_02380	*groL*	chaperone peptide-dependent heat shock protein	2.13276	<0.001
NF29_07145	*arnT*	4-amino-4-deoxy-L-arabinose transferase (lipid A modification)	2.1231	0.00115446
NF29_13400	*btuB*	outer membrane receptor for transport of vitamin e and bacteriophage bf23	2.1083	<0.001
NF29_04305	*ggt*	gamma-glutamyltranspeptidase	2.07919	<0.001
NF29_15595	*osmF*	transport system permease protein	2.05157	0.00104216
NF29_05360	*blc*	outer membrane lipoprotein	2.03672	<0.001
NF29_18530	*katE*	catalase hydroperoxidase hpii	2.03013	<0.001
NF29_21570	*None*	---NA---	2.0148	0.021
NF29_21965	*potG*	atp-binding component of a transport system	2.01086	<0.001
NF29_04505	*yjbE*	extracellular polysaccharide production threonine-rich protein	1.99874	<0.001
NF29_14505	*fhuA*	outer membrane protein receptor for colicin and phages and phi80	1.99125	<0.001
NF29_20165	*ybjX*	putative enzyme	1.98739	<0.001

NF29_21670	*fepG*	ferric enterobactin transport protein	1.95755	0.000842263
NF29_21975	*None*	---NA---	1.95544	<0.001
NF29_21195	*kdpB*	atpase of high-affinity potassium transport b chain	1.94874	<0.001
NF29_17245	*emrY*	transport protein	1.93453	<0.001
NF29_20625	cusC	resistance protein	1.92689	0.037
NF29_09365	*None*	---NA---	1.92328	<0.001
NF29_21695	*fepA*	outer membrane receptor for ferric enterobactin and colicins b and d	1.92291	<0.001
NF29_19340	*yhhW*	quercetin 2,3-dioxygenase	1.92266	0.017486
NF29_04250	*yjbJ*	stress-induced protein	1.89618	<0.001
NF29_21970	*cysU*	thiosulfate transport system permease t protein	1.89596	<0.001
NF29_18950	*ycfJ*	hypothetical protein	1.88193	<0.001
NF29_13090	*wcaM*	colanic acid biosynthesis protein	1.85449	<0.001
NF29_09180	*fecD*	citrate-dependent iron membrane-bound protein	1.85136	0.0394595
NF29_13025	*wcaD*	colanic acid polymerase	1.8396	<0.001
NF29_11690	*talB*	transaldolase a	1.83033	<0.001
NF29_19370	*efeO*	ferrous ion transporter	1.82861	<0.001
NF29_19630	*mhpC*	2-hydroxy-6-ketonona- -dienedioic acid hydrolase	1.82592	<0.001
NF29_16880	*glgX*	part of glycogen a glycosyl debranching enzyme	1.82504	0.00358073
NF29_11460	*rbsA*	atp-binding component of d-ribose high-affinity transport system	1.80831	0.0131072
NF29_04490	*yjbH*	DUF940 family extracellular polysaccharide protein	1.79045	<0.001
NF29_11890	*ilvI*	acetolactate synthase valine large subunit	1.78936	<0.001
NF29_12775	*yehY*	transport system permease protein	1.78119	0.00946137
NF29_16235	*glpR*	deor-type transcriptional regulator	1.77526	<0.001
NF29_15505	*None*	---NA---	1.77378	0.00158244
NF29_22205	*htpG*	chaperone heat shock protein c	1.75733	<0.001
NF29_13760	*None*	---NA---	1.75458	<0.001
NF29_11680	*ypfG*	hypothetical protein	1.73647	<0.001
NF29_20200	*poxB*	pyruvate oxidase	1.71714	<0.001
NF29_18275	*sufB*	Fe-S cluster assembly protein	1.71448	<0.001
NF29_14500	*ymgE*	hypothetical protein	1.71149	<0.001
NF29_19860	*None*	---NA---	1.68455	0.0211008
NF29_20780	*ybhF*	atp-binding component of a transport system	1.68277	0.00165022
NF29_19610	*None*	---NA---	1.66892	<0.001
NF29_14930	*ychO*	attaching and effacing pathogenesis factor	1.65712	<0.001

NF29_21675	*fepC*	atp-binding component of a transport system	1.64404	0.0025672 4
NF29_21680	*entF*	atp-dependent serine activating enzyme (may be part of enterobactin synthase as component f)	1.64322	<0.001
NF29_19625	*None*	---NA---	1.6256	0.0017488 5
NF29_01040	*None*	---NA---	1.61667	<0.001
NF29_03990	*ybdR*	threonine dehydrogenase	1.60901	<0.001
NF29_20775	*ybhG*	membrane protein	1.60595	0.0484751
NF29_10510	*srlD*	glucitol -6-phosphate dehydrogenase	1.58928	0.0231663
NF29_19865	*None*	---NA---	1.58214	<0.001
NF29_12140	*yfcG*	thiol:disulfide oxidoreductase	1.57743	0.0239814
NF29_01310	*sad*	aldehyde dehydrogenase	1.57739	<0.001
NF29_21655	*fepB*	ferric enterobactin binding protein periplasmic component	1.57563	0.0061090 5
NF29_17475	*ydcS*	transport protein	1.56135	0.0233201
NF29_20620	*yajO*	nad h-dependent xylose reductase	1.55861	0.0200753
NF29_14510	*treA*	cytoplasmic trehalase	1.55133	<0.001
NF29_15135	*tonB*	energy transducer uptake of cyanocobalimin sensitivity to colicins	1.55071	<0.001
NF29_14475	*dadX*	alanine racemase catabolic	1.52918	0.0034273 6
NF29_00430	yacH	putative membrane protein	1.5079	<0.001
NF29_10965	*None*	---NA---	1.50539	0.0016674 4
NF29_00850	*nhaA*	na+ h antiporter ph dependent	1.5053	<0.001
NF29_09185	*fepB*	ferric enterobactin binding protein periplasmic component	1.50446	0.012397
NF29_04335	*ugpB*	sn-glycerol 3-phosphate transport system periplasmic binding protein	1.48352	0.0218179
NF29_19855	*None*	---NA---	1.47899	<0.001
NF29_13305	*amn*	amp nucleosidase	1.47007	<0.001
NF29_16185	*None*	---NA---	1.45899	<0.001
NF29_17095	*sra*	30s ribosomal subunit protein s22	1.43787	<0.001
NF29_17400	*curA*	NADP-dependent oxidoreductase	1.43614	0.0007388 71
NF29_16870	*glgX*	part of glycogen a glycosyl debranching enzyme	1.42887	0.0014566 5
NF29_17685	*ycjQ*	alcohol dehydrogenase class iii formaldehyde glutathione-dependent	1.41998	0.02952
NF29_13865	*otsA*	trehalose-6-phosphate synthase	1.41951	<0.001
NF29_04820	*yiaD*	outer membrane protein	1.41641	<0.001
NF29_12925	*yegP*	hypothetical protein	1.40893	<0.001
NF29_21110	*sucC*	succinyl- beta subunit	1.4085	0.007
NF29_12040	*yfcJ*	transport protein	1.40538	0.0200254

NF29_09250	*dkgA*	aldose reductase	1.39884	<0.001
NF29_14460	*ycgB*	sporulation protein	1.38271	<0.001
NF29_09385	*None*	---NA---	1.38087	<0.001
NF29_22355	*ybaY*	glycoprotein polysaccharide metabolism	1.37516	<0.001
NF29_16600	*marA*	arac-type regulatory protein	1.37482	<0.001
NF29_01045	*osmY*	hyperosmotically inducible periplasmic protein	1.37188	<0.001
NF29_10840	*None*	---NA---	1.3649	<0.001
NF29_17860	*acnA*	aconitate hydrase 1	1.36303	<0.001
NF29_11880	*ypeC*	hypothetical protein	1.35438	<0.001
NF29_16885	*None*	---NA---	1.34908	0.0062090 4
NF29_20330	*ybjP*	lipoprotein	1.34486	<0.001
NF29_06875	*treF*	cytoplasmic trehalase	1.33979	<0.001
NF29_19880	*None*	---NA---	1.33877	<0.001
NF29_19620	*entF*	atp-dependent serine activating enzyme (may be part of enterobactin synthase as component f)	1.3371	0.0047005 3
NF29_18270	*sufC*	atp-binding component of a transport system	1.33522	0.035491
NF29_22535	*yajO*	nad h-dependent xylose reductase	1.32582	<0.001
NF29_19490	*yqjA*	general envelope maintenance protein	1.32495	0.0013088 4
NF29_18490	*pfkB*	6-phosphofructokinase ii suppressor of pfka	1.32365	0.0476518
NF29_05830	*rbsC*	transport system permease protein	1.32357	<0.001
NF29_20630	*emrB*	transport protein	1.32344	0.0198526
NF29_02385	*groS*	10 kd chaperone binds to hsp60 in mg-suppressing its atpase activity	1.32283	<0.001
NF29_16350	*gcd*	glucose dehydrogenase	1.32221	0.0080494 7
NF29_13260	*dacD*	penicillin binding protein 6b	1.32168	<0.001
NF29_12720	*bglF*	pts system beta- enzyme cryptic	1.31898	0.0452603
NF29_18345	*ppsA*	pep-protein phosphotransferase system enzyme i	1.31821	<0.001
NF29_00305	*fhuA*	outer membrane protein receptor for colicin and phages and phi80	1.31262	<0.001
NF29_08835	*patA*	acetylornithine delta-aminotransferase	1.30479	<0.001
NF29_16340	*frmA*	alcohol dehydrogenase class iii formaldehyde glutathione-dependent	1.30437	0.0144234
NF29_10790	*yeaM*	arac-type regulatory protein	1.29356	0.0416457
NF29_13860	otsB	trehalose-6-phosphate biosynthetic	1.29098	0.027
NF29_17240	*kup*	low affinity potassium transport system	1.28388	0.0052059
NF29_17385	*yncE*	hypothetical protein	1.28269	<0.001
NF29_15345	*fumC*	fumarase c= fumarate hydratase class ii isozyme	1.27359	0.0134729
NF29_02055	*msrA*	peptide methionine sulfoxide reductase	1.26126	0.0007960 86

NF29_12375	*glpT*	sn-glycerol-3-phosphate permease	1.26045	<0.001
NF29_06190	*hslV*	heat shock protein proteasome-related peptidase subunit	1.25423	<0.001
NF29_03145	*fmrR*	alpha helix chain	1.25337	0.0030433 4
NF29_17110	*adhP*	alcohol dehydrogenase	1.25113	<0.001
NF29_08215	*dhaK*	dihydroxyacetone kinase	1.24836	<0.001
NF29_15545	*None*	---NA---	1.24585	<0.001
NF29_08105	*gabD*	succinate-semialdehyde nadp-dependent activity	1.23954	<0.001
NF29_10355	*mdtH*	transport protein	1.23783	0.0348671
NF29_14035	*yecD*	isochorismatase family protein	1.23039	0.0018016 2
NF29_18250	*ldtE*	transpeptidase	1.22806	0.0012491
NF29_14290	*None*	---NA---	1.21968	<0.001
NF29_00425	*acnB*	aconitate hydrase b	1.21619	<0.001
NF29_18725	*yeaG*	serine/threonine protein kinase	1.21496	<0.001
NF29_18260	*sufS*	cysteine desulfurase	1.20541	0.0135879
NF29_16225	*cybB*	cytochrome b	1.2025	0.0006303 35
NF29_06890	*prlC*	oligopeptidase a	1.19999	<0.001
NF29_00665	*fieF*	putative transport system permease protein	1.1981	<0.001
NF29_00900	*yaaX*	hypothetical protein	1.19061	0.0337188
NF29_12175	*ulaA*	ascorbate-specific permease IIC component	1.17932	0.011796
NF29_13340	*mgtA*	mg2+ transport p-type 1	1.17928	0.0080278 9
NF29_18940	*bhsA*	biofilm, cell surface and signaling protein	1.17043	<0.001
NF29_15800	*None*	---NA---	1.16945	0.0114468
NF29_01005	*deoB*	phosphopentomutase	1.15831	<0.001
NF29_11695	*maeB*	multimodular enzyme	1.15798	<0.001
NF29_10150	*gudD*	glucarate dehydratase	1.15684	0.011796
NF29_02025	*None*	---NA---	1.15608	0.0230898
NF29_20725	*ybiJ*	hypothetical protein	1.15453	<0.001
NF29_07955	*yhdV*	membrane protein	1.15365	0.0343176
NF29_19615	*None*	---NA---	1.14888	0.0357013
NF29_10690	*ygaC*	hypothetical protein	1.14868	<0.001
NF29_12635	*cirA*	outer membrane receptor for iron-regulated colicin i receptor porin requires tonb gene product	1.14829	0.0126626
NF29_11185	*murP*	pts enzyme ii	1.14528	0.0153769
NF29_10665	*nrdF*	ribonucleoside-diphosphate reductase beta frag	1.14203	0.025
NF29_15670	*acrF*	integral transmembrane protein acridine resistance	1.13896	0.010331
NF29_00135	*ldcC*	lysine decarboxylase constitutive	1.13434	<0.001

NF29_07400	*yhgF*	30s ribosomal subunit protein s1	1.12657	<0.001
NF29_06620	*yigM*	putative inner membrane EamA-like transporter	1.11351	0.0248536
NF29_00855	*dnaJ*	chaperone with heat shock protein	1.1123	<0.001
NF29_12285	*nuoL*	hydrogenase 4 membrane subunit	1.11039	0.0260755
NF29_18730	*yeaH*	hypothetical protein	1.10578	<0.001
NF29_11260	*None*	---NA---	1.10303	0.0012115 9
NF29_00805	carA	carbamoyl-phosphate glutamine subunit	1.09651	<0.001
NF29_21115	*sucB*	2-oxoglutarate dehydrogenase (dihydrolipoyltranssuccinase e2 component)	1.09252	0.0007198 99
NF29_20810	*ybhN*	hypothetical protein	1.09024	0.0494455
NF29_11320	*pepB*	aminopeptidase a i	1.08838	0.0010423 7
NF29_01100	*fhuF*	ferric iron reductase involved in ferric hydroximate transport	1.08493	<0.001
NF29_20670	*dps*	Fe-binding and storage protein; stress-inducible DNA binding protein	1.07932	0.0018448
NF29_04435	*zntA*	zinc-transporting atpase	1.0759	0.0041138 8
NF29_17265	*fusA*	gtp-binding protein chain elongation factor ef-g	1.07352	0.0009243 65
NF29_06185	*hslU*	heat shock protein atpase homologous to chaperones	1.07091	<0.001
NF29_04430	*yhhN*	membrane protein	1.06487	0.0021659 4
NF29_16760	*None*	---NA---	1.06311	<0.001
NF29_22715	*phoA*	alkaline phosphatase	1.04282	0.0177967
NF29_21095	*mngA*	protein modification induction of ompc	1.04117	0.0494455
NF29_01255	*rhmT*	transport protein	1.04005	0.0477068
NF29_12260	*nuoG*	nadh dehydrogenase i chain g	1.03827	0.0098444 1
NF29_08755	*terC*	transport protein	1.03742	0.021
NF29_14345	*manY*	pts enzyme mannose-specific	1.03419	0.0010663 7
NF29_11030	*clpB*	heat shock protein	1.03052	0.0017111 3
NF29_17070	*osmC*	osmotically inducible protein	1.02328	0.0007626 45
NF29_16220	*gapA*	glyceraldehyde-3-phosphate dehydrogenase a	1.01508	0.0397796
NF29_00685	*sgrR*	transport protein	1.00938	0.0075818 9
NF29_10370	*None*	---NA---	1.00736	0.0466146
NF29_05720	*pstS*	high-affinity phosphate-specific transport system periplasmic phosphate-binding protein	1.00536	0.0012668 8
NF29_11335	*sseA*	thiosulfate sulfurtransferase	1.0045	0.0405085
NF29_10905	*recN*	protein used in recombination and dna repair	1.00095	0.0011713 7
NF29_02680	*None*	---NA---	1.00018	0.0245871

NF29_12380	*glpQ*	glycerophosphodiester periplasmic	0.998362	0.0036663 1
NF29_02340	*blc*	outer membrane lipoprotein	0.997642	0.0054966 6
NF29_20340	*artI*	arginine 3rd transport system periplasmic binding protein	0.99378	0.0011082 3
NF29_00845	*nhaR*	transcriptional regulator lysr-type	0.985432	0.0014245 1
NF29_20900	*mngR*	transcriptional regulator	0.978152	0.0261676
NF29_13545	*rcsA*	positive regulator for ctr capsule positive transcription factor	0.974131	0.0065684 7
NF29_19395	*putA*	aldehyde dehydrogenase	0.967466	0.0042731 9
NF29_16665	*yaiC*	sensor-type protein	0.965685	0.013304
NF29_18280	*sufA*	Fe-S cluster assembly protein	0.960938	0.0225183
NF29_09195	*ygaU*	hypothetical protein	0.959918	0.0026967 8
NF29_01010	*deoA*	thymidine phosphorylase	0.957349	0.017698
NF29_09255	*yqhD*	ethanolamine utilization homolog of salmonella iron-containing alcohol dehydrogenase	0.953325	0.031
NF29_16245	*ORF_o222*	hypothetical protein	0.951608	0.0067032 7
NF29_01090	*None*	---NA---	0.947091	0.0024609 3
NF29_18420	*ydiU*	hypothetical protein	0.944674	0.0114814
NF29_07255	*yhhT*	transport protein	0.942022	0.0324509
NF29_04775	*dppB*	transport system permease protein	0.93906	0.0137907
NF29_16895	*None*	---NA---	0.935135	0.0163526
NF29_22765	*ampH*	beta-lactamase penicillin resistance	0.930069	0.0337188
NF29_00470	*yagG*	glucuronide permease	0.928143	0.0253747
NF29_01985	fbp	fructose-1,6-bisphosphatase I	0.927548	0.005
NF29_07305	*glgC*	glucose-1-phosphate thymidylyltransferase	0.922654	0.0146679
NF29_14340	*manZ*	pts enzyme mannose-specific	0.916183	0.0051827
NF29_21870	*mgtA*	mg2+ transport p-type 1	0.909424	0.0042307 2
NF29_01870	*None*	---NA---	0.900605	0.012397
NF29_15365	*rstA*	response transcriptional regulatory protein (sensor)	0.899441	0.0401194
NF29_00435	*None*	hypothetical protein	0.89336	0.0452223
NF29_00445	*aceF*	pyruvate dehydrogenase (dihydrolipoyltransacetylase component)	0.892621	0.0075779 7
NF29_04310	*None*	---NA---	0.891442	0.0144234
NF29_14580	*None*	---NA---	0.889346	0.0075702 1
NF29_07390	*feoB*	ferrous iron transport protein b	0.884113	0.0074742
NF29_00800	*carB*	carbamoyl-phosphate synthase large subunit	0.882134	0.0061367

NF29_22685	*yaiA*	hypothetical protein	0.881186	0.00733504
NF29_21205	*kdpD*	sensor for high-affinity potassium transport system	0.876524	0.0441286
NF29_16700	*None*	---NA---	0.87574	0.017018
NF29_18585	*spy*	periplasmic protein related to spheroblast formation	0.87099	0.022
NF29_21270	*nagE*	pts n-acetylglucosamine-specific enzyme iiabc	0.87041	0.00892304
NF29_01855	*pyrB*	aspartate catalytic subunit	0.865969	0.00977052
NF29_13535	*yodB*	hypothetical protein	0.864506	0.0476518
NF29_21715	*betB*	nad+-dependent betaine aldehyde dehydrogenase	0.859669	0.0174216
NF29_07120	*None*	---NA---	0.85041	0.0109307
NF29_12480	*yajR*	transport protein	0.847991	0.0114468
NF29_21885	*None*	---NA---	0.844488	0.00911225
NF29_01290	*yjiJ*	metal-binding GTPase	0.840765	0.00911225
NF29_13335	*uspG*	filament protein	0.83288	0.0250078
NF29_12910	*yegS*	phosphatidylglycerol kinase metal-dependent	0.832667	0.0156956
NF29_02160	*yjfY*	hypothetical protein	0.829589	0.0129393
NF29_18900	*cobB*	nicotinic acid mononucleotide: -dimethylbenzimidazole phosphoribosyltransferase	0.828413	0.0192734
NF29_22925	*yaiV*	putative transcriptional regulator	0.822457	0.0118042
NF29_21120	*sucA*	2-oxoglutarate dehydrogenase (decarboxylase component)	0.821868	0.0189167
NF29_12770	*osmF*	transport system permease protein	0.8206	0.0337726
NF29_22505	*panE*	involved in thiamin alternative pyrimidine biosynthesis	0.814279	0.022889
NF29_21960	*yahO*	hypothetical protein	0.813713	0.0125039
NF29_03150	*fmrA*	alcohol dehydrogenase class iii formaldehyde glutathione-dependent	0.813543	0.0118546
NF29_04990	*mtlA*	pts mannitol-specific enzyme iiabc components	0.806934	0.0178866
NF29_12740	*yohD*	DedA family Inner membrane protein	0.806535	0.0143082
NF29_22255	*acrA*	membrane protein	0.803824	0.025422
NF29_07360	*gntP*	high-affinity transport of gluconate gluconate permease	0.801483	0.041
NF29_13630	*amyA*	cytoplasmic alpha-amylase	0.801143	0.0324196
NF29_08210	*yjgR*	hypothetical protein	0.792627	0.0308215
NF29_22390	*cysM*	cysteine synthase o-acetylserine sulfhydrolase b	0.790252	0.0179159
NF29_21720	*betA*	choline a flavoprotein	0.787295	0.0205571
NF29_12990	*yegH*	transport protein	0.786646	0.0310697
NF29_12760	*dld*	d-lactate fad nadh independent	0.780696	0.0162865

NF29_13265	*sbmC*	DNA gyrase inhibitor	0.780381	0.0235752
NF29_19240	*mysB*	acidic protein suppresses mutants lacking function of protein export	0.779668	0.0174216
NF29_01280	cstA	carbon starvation protein	0.772438	0.028
NF29_02335	*ampC*	beta-lactamase penicillin resistance	0.772121	0.0478137
NF29_02015	*ytfQ*	laci-type transcriptional regulator	0.771716	0.0339596
NF29_13300	*ypdF*	aminopeptidase	0.76922	0.0315635
NF29_12765	*bglX*	beta-d-glucoside periplasmic	0.768687	0.0186806
NF29_09330	*yghU*	putative S-transferase	0.763663	0.0204871
NF29_14305	*None*	---NA---	0.757538	0.0357013
NF29_14470	*dadA*	d-amino acid dehydrogenase subunit	0.755606	0.033344
NF29_20335	*artP*	atp-binding component of a transport system	0.751241	0.022147
NF29_06840	*yhjG*	hypothetical protein	0.744233	0.0249741
NF29_17520	*ompN*	outer membrane protein	0.740507	0.0309671
NF29_18165	*ydhS*	oxidoreductase	0.739053	0.0401194
NF29_14230	*ybjX*	putative enzyme	0.729342	0.0340648
NF29_14585	*None*	---NA---	0.724281	0.0281026
NF29_14865	*chaB*	cation transport regulator	0.721401	0.0468289
NF29_15180	*narP*	nitrate nitrite response regulator (sensor)	0.716718	0.039
NF29_04180	*yjbR*	hypothetical protein	0.715429	0.0466081
NF29_19365	*efeB*	deferrrochelatase, periplasmic	0.712004	0.0337188
NF29_14235	*pphA*	protein phosphatase 1 modulates signals protein misfolding	0.708311	0.0336352
NF29_04780	*dppA*	dipeptide transport protein	0.705039	0.0466247
NF29_08705	*yqjE*	hypothetical protein	0.703604	0.0440834
NF29_12675	*mglC*	transport system permease protein	0.702629	0.0433655
NF29_21125	*sdhB*	succinate iron sulfur protein	0.698316	0.0379397
NF29_14645	*None*	---NA---	0.697571	0.0487165
NF29_06255	*katG*	catalase hydroperoxidase hpi	0.690655	0.0476518
NF29_05825	*rbsA*	atp-binding component of d-ribose high-affinity transport system	0.680732	0.0493609
NF29_10970	*yfiB*	outer membrane protein	-0.684748	0.0487456
NF29_16610	*marC*	channel protein	-0.692812	0.0452603
NF29_14890	*None*	---NA---	-0.694959	0.0468289
NF29_09635	*serA*	d-3-phosphoglycerate dehydrogenase	-0.700443	0.0414254
NF29_19715	*yccF*	hypothetical protein	-0.706155	0.041719
NF29_08320	*yrbG*	hypothetical protein	-0.707211	0.0417103
NF29_14260	*msrC*	free methionine-(R)-sulfoxide reductase	-0.707768	0.0398597
NF29_00005	*mltD*	transcriptional regulator for nitrite reductase (cytochrome c552)	-0.715224	0.0416443
NF29_19220	*yceI*	hypothetical protein	-0.716885	0.0357014

NF29_15130	*yciI*	putative DGPF domain-containing enzyme	-0.718207	0.0342599
NF29_06535	*cyaA*	adenylate cyclase	-0.721159	0.0484751
NF29_06260	*gldA*	glycerol dehydrogenase	-0.721278	0.033
NF29_22250	*acrR*	acrab operon repressor	-0.722562	0.0407741
NF29_08495	*deaD*	atp-dependent rna helicase	-0.724463	0.0357014
NF29_03665	*None*	---NA---	-0.72586	0.0337188
NF29_05200	*slmA*	nucleoid occlusion factor	-0.72682	0.0324509
NF29_04715	*nusG*	component in transcription antitermination	-0.728496	0.0453403
NF29_12210	*yfbT*	sugar phosphatase	-0.731067	0.040347
NF29_06590	*pldA*	outer membrane phospholipase a	-0.732358	0.0324509
NF29_02205	*nsrR*	nitric oxide-sensitive repressor for NO regulon	-0.733229	0.0317536
NF29_18790	yaiC	diguanylate cyclase	-0.73388	0.039
NF29_06545	*None*	---NA---	-0.741395	0.0324196
NF29_17845	*yciS*	hypothetical protein	-0.742242	0.0346119
NF29_05925	*hemN*	o2-independent coproporphyrinogen iii oxidase	-0.745698	0.0327062
NF29_07420	*pck*	phosphoenolpyruvate carboxykinase	-0.747995	0.0447646
NF29_13725	*pgsA*	phosphatidylglycerophosphate synthetase = cdp- -diacyl-sn-glycero-3-phosphate phosphatidyl transferase	-0.749473	0.0347131
NF29_06170	*zapB*	FtsZ stabilizer	-0.750235	0.0465921
NF29_09085	*ygiM*	signal transduction protein	-0.750944	0.035491
NF29_03045	*None*	---NA---	-0.754399	0.0388215
NF29_10180	*pyrG*	ctp synthetase	-0.755846	0.0445468
NF29_15070	*cisA*	cardiolipin a major membrane phospholipid novobiocin sensitivity	-0.756557	0.0264126
NF29_05610	*yidA*	sugar phosphate phosphatase	-0.756712	0.0323119
NF29_03190	*None*	---NA---	-0.757418	0.0234473
NF29_15470	*None*	---NA---	-0.758377	0.0323119
NF29_13685	*fliY*	arginine 3rd transport system periplasmic binding protein	-0.760028	0.0349144
NF29_11570	*pepA*	aminopeptidase a i	-0.764043	0.028
NF29_12525	*yejG*	hypothetical protein	-0.764485	0.0248404
NF29_08175	*sspA*	regulator of transcription stringent starvation protein a	-0.764748	0.0437235
NF29_22500	*yajQ*	nucleotide-binding protein	-0.765185	0.0439125
NF29_13620	*None*	---NA---	-0.768072	0.0285825
NF29_09600	*pgk*	phosphoglycerate kinase	-0.768133	0.0467379
NF29_12805	*yehS*	hypothetical protein	-0.768355	0.0210217
NF29_11625	*purC*	phosphoribosylaminoimidazole-succinocarboxamide synthetase = saicar synthetase	-0.770327	0.0323119
NF29_07040	*yobA*	hypothetical protein	-0.770952	0.0259146

NF29_06955	*None*	---NA---	-0.77119	0.0221668
NF29_07615	*None*	---NA---	-0.772497	0.0221668
NF29_07850	*trkA*	transport of potassium	-0.772804	0.0218179
NF29_10660	*proV*	atp-binding component of a transport system	-0.773258	0.0452223
NF29_07365	*nfuA*	Fe-S biogenesis protein	-0.773813	0.029173
NF29_05045	*secB*	protein export molecular chaperone may bind to signel sequence	-0.774805	0.0428646
NF29_00930	*creA*	putative periplasmic protein	-0.776021	0.0235752
NF29_03220	*None*	---NA---	-0.776704	0.0203026
NF29_05910	*polA*	dna polymerase 3 --	-0.777196	0.0313963
NF29_20840	*moaA*	molybdopterin protein a	-0.778318	0.0208635
NF29_01110	*ybaK*	hypothetical protein	-0.780943	0.0192734
NF29_09520	*yggS*	UPF0001 family protein, PLP-binding	-0.78122	0.0194688
NF29_22675	*yaiE*	hypothetical protein	-0.781844	0.0209655
NF29_10640	*mprA*	regulator of plasmid mcrb operon (microcin b17 synthesis)	-0.781989	0.027
NF29_08535	*yhbP*	hypothetical protein	-0.783153	0.0306264
NF29_04035	*yfcI*	transposase_31 family protein	-0.784048	0.0358091
NF29_16290	*yhjC*	transcriptional regulator lysr-type	-0.784109	0.0206115
NF29_11765	*ptsI*	pep-protein phosphotransferase system enzyme i	-0.788765	0.0420944
NF29_19945	*smtA*	s-adenosylmethionine-dependent methyltransferase	-0.789279	0.0210579
NF29_13980	*sfmA*	fimbrial-like protein	-0.78929	0.0333643
NF29_17885	*topA*	dna topoisomerase type omega protein	-0.791933	0.0276591
NF29_01910	*relB*	negative regulator of translation	-0.793024	0.047784
NF29_08365	ispB	octaprenyl diphosphate synthase	-0.793841	0.024
NF29_08385	*obgE*	gtp-binding factor	-0.796123	0.0260986
NF29_09675	*gcvH*	in glycine cleavage carrier of aminomethyl moiety via covalently bound lipoyl cofactor	-0.796513	0.0309063
NF29_02150	*priB*	primosomal replication protein n	-0.797055	0.0329478
NF29_05730	*glmU*	n-acetyl glucosamine-1-phosphate uridyltransferase	-0.797085	0.0275214
NF29_20035	*pflA*	pyruvate formate lyase activating enzyme 1	-0.799589	0.0277362
NF29_22130	*fetB*	metal resistance protein	-0.801842	0.0254135
NF29_06350	*yifA*	regulator of pssa	-0.801894	0.0218179
NF29_11990	*sixA*	phosphohistidine phosphatase	-0.806482	0.0151882
NF29_15140	*yciA*	acyl-CoA esterase	-0.808592	0.0151976
NF29_14830	*hemA*	enzyme in alternate path of synthesis of 5-aminolevulinate	-0.81067	0.020488
NF29_08500	*mtr*	tryptophan-specific transport protein	-0.810735	0.0203026
NF29_08025	*None*	---NA---	-0.819321	0.0212622
NF29_00835	*ribF*	bifunctional riboflavin kinase/FAD synthetase	-0.819456	0.0151976

NF29_14400	*yeaY*	outer membrane protein	-0.820986	0.0163965
NF29_14285	*kdgR*	transcriptional regulator	-0.822474	0.025
NF29_04260	*lexA*	regulator for sos regulon	-0.823662	0.0246515
NF29_13190	*rfb*	dtdp-glucose dehydratase	-0.823991	0.0212622
NF29_19195	*bssS*	biofilm regulator	-0.826038	0.0210656
NF29_07845	*mscL*	mechanosensitive channel	-0.827829	0.0248679
NF29_12205	*yfbU*	hypothetical protein	-0.828502	0.0218179
NF29_09850	*citA*	sensor-type protein	-0.828602	0.0220471
NF29_02310	*frdA*	fumarate flavoprotein subunit	-0.830201	0.029173
NF29_21245	*fldA*	flavodoxin 1	-0.831137	0.0218179
NF29_22200	*adk*	adenylate kinase activity pleiotropic effects on glycerol-3-phosphate acyltransferase activity	-0.834034	0.0167199
NF29_18710	*gapA*	glyceraldehyde-3-phosphate dehydrogenase a	-0.834137	0.0307105
NF29_04375	*panM*	panothenate synthesis	-0.835848	0.012584
NF29_19995	*rpsA*	30s ribosomal subunit protein s1	-0.835921	0.0323119
NF29_02800	*None*	---NA---	-0.836895	0.012016
NF29_18560	*chbB*	pep-dependent phosphotransferase enzyme iv for and salicin	-0.838636	0.0115434
NF29_06100	*cpxR*	transcriptional regulator in 2-component system	-0.839545	0.0247963
NF29_08060	*mreD*	rod shape-determining protein	-0.844723	0.0477496
NF29_15940	*btuE*	vitamin b12 transport	-0.847785	0.023839
NF29_06395	*ilvC*	ketol-acid reductoisomerase	-0.851082	0.0189606
NF29_09930	*ascG*	ascbf operon repressor	-0.854622	0.0150746
NF29_18470	*rpmI*	50s ribosomal subunit protein a	-0.857716	0.023839
NF29_03310	*None*	---NA---	-0.858574	0.00995521
NF29_19845	*cohE*	phage repressor	-0.861523	0.019
NF29_10695	*alaE*	alanine exporter, alanine-inducible, stress responsive	-0.863036	0.0108295
NF29_09860	*yjdJ*	GNAT family putative N-acetyltransferase	-0.863425	0.00852428
NF29_08995	*dnaG*	dna biosynthesis dna primase	-0.863658	0.0102981
NF29_06660	*ubiB*	ubiquinone biosynthesis protein	-0.863746	0.0172453
NF29_11290	*iscU*	iron-sulfur cluster assembly scaffold protein	-0.863753	0.0129529
NF29_19065	*rluC*	pseudouridylate synthase	-0.86721	0.00976585
NF29_02285	*orn*	oligoribonuclease	-0.870153	0.00712133
NF29_22585	*secF*	protein membrane protein	-0.873073	0.0115434
NF29_01715	None	---NA---	-0.875653	0.016
NF29_11305	*hscA*	heat shock member of hsp70 protein family	-0.876531	0.00670327
NF29_09855	*yjdI*	putative 4Fe-4S mono-cluster protein	-0.881776	0.0115434

NF29_11015	*bamD*	BamABCDE complex OM biogenesis lipoprotein	-0.882249	0.0135879
NF29_20010	*serC*	3-phosphoserine aminotransferase	-0.883788	0.0115434
NF29_07630	*fkpA*	fkbp-type peptidyl-prolyl cis-trans isomerase	-0.884258	0.0166778
NF29_04200	*nadR*	probable nadab transcriptional regulator	-0.886704	0.0225183
NF29_07825	*rplQ*	50s ribosomal subunit protein l17	-0.88675	0.0135095
NF29_12495	*yejL*	hypothetical protein	-0.88699	0.0062090 4
NF29_06400	*ppiC*	peptidyl-prolyl cis-trans isomerase c (rotamase c)	-0.88833	0.012323
NF29_21430	*mrdB*	rod shape-determining membrane protein sensitivity to radiation and drugs	-0.88896	0.0077820 2
NF29_13375	*None*	---NA---	-0.89032	0.0061124 1
NF29_04510	*pgi*	glucosephosphate isomerase	-0.892992	0.012616
NF29_07900	*yrdA*	possible synthesis of cofactor for carnitine racemase and dehydratase	-0.89705	0.034727
NF29_07620	*slyD*	fkbp-type peptidyl-prolyl cis-trans isomerase	-0.897873	0.0121285
NF29_02235	*hfq*	host factor i for bacteriophage q beta a growth-related protein	-0.900135	0.013
NF29_00215	*tsf*	protein chain elongation factor ef-ts	-0.902916	0.0146268
NF29_07820	*rpoA*	rna alpha subunit	-0.903491	0.0155578
NF29_06480	*wecG*	probable udp-n-acetyl-d-mannosaminuronic acid transferase synthesis of enterobacterial common antigen	-0.905286	0.0054625 4
NF29_22370	*glnK*	nitrogen regulatory protein p-ii 2	-0.906471	0.0323119
NF29_17820	*yciT*	deor-type transcriptional regulator	-0.906741	0.0052059
NF29_22610	*acpH*	acyl carrier protein (ACP) phosphodiesterase	-0.908025	0.0169477
NF29_01710	*None*	---NA---	-0.908798	0.0056161 2
NF29_06670	*tatB*	TatABCE protein translocation system subunit	-0.9096	0.0076608
NF29_19960	*kdsB*	ctp:cmp-3-deoxy-d-manno-octulosonate transferase	-0.911629	0.0204871
NF29_08155	*rplM*	50s ribosomal subunit protein l13	-0.911908	0.0117809
NF29_02240	*miaA*	delta -isopentenylpyrophosphate trna-adenosine transferase	-0.912894	0.0127274
NF29_00195	*ispU*	undecaprenyl pyrophosphate synthase	-0.914785	0.0084141 1
NF29_05075	*pgaC*	biofilm PGA synthase	-0.915066	0.0074742
NF29_17540	*None*	---NA---	-0.918924	0.0049311 6
NF29_01145	*osmC*	osmotically inducible protein	-0.92156	0.0052059
NF29_08145	*yhcB*	hypothetical protein	-0.922279	0.0115434
NF29_08065	*yhdE*	dTTP/UTP pyrophosphatase	-0.922297	0.0437558
NF29_11145	*rnc*	rnase ds rna	-0.92374	0.02255
NF29_15760	*ydfZ*	polynucleotide phosphorylase/polyadenylase	-0.92466	0.0044648 8

NF29_08485	*pnp*	polynucleotide phosphorylase cytidylate kinase activity	-0.925074	0.0112051
NF29_00745	*djlA*	dna binding protein	-0.925955	0.0047694
NF29_03780	*None*	---NA---	-0.926261	0.004
NF29_06275	*ppc*	phosphoenolpyruvate carboxylase	-0.928787	0.0069849 7
NF29_00540	*secM*	regulator of secA translation	-0.930341	0.0151882
NF29_00220	*rpsB*	30s ribosomal subunit protein s2	-0.935624	0.0102964
NF29_18505	*yniB*	putative inner membrane protein	-0.936601	0.0038601
NF29_04485	*lacA*	thiogalactoside acetyltransferase	-0.937362	0.0041138 8
NF29_23150	*None*	---NA---	-0.942712	0.0157434
NF29_04610	*hupA*	dna-binding protein hu-alpha (hu-2)	-0.943937	0.0108812
NF29_17855	*ribA*	gtp cyclohydrolase ii	-0.944953	0.0051827
NF29_21065	ybgC	acyl-CoA thioester hydrolase	-0.950737	0.006
NF29_06685	*rfaH*	transcriptional activator affecting biosynthesis of lipopolysaccharide f and haemolysin	-0.952412	0.0056088
NF29_18475	*infC*	protein chain initiation factor if-3	-0.952994	0.0096805
NF29_05245	*trmH*	rna methylase	-0.954314	0.0075702 1
NF29_08645	*tdcA*	transcriptional regulator lysr-type	-0.955986	0.0063902 3
NF29_15530	*mdtJ*	possible chaperone	-0.956124	0.0038575 8
NF29_04855	*yiaF*	barrier effect co-colonization resistance factor	-0.956597	0.0050610 4
NF29_19915	*ycbL*	probable hydroxyacylglutathione hydrolase	-0.962682	0.0044152 9
NF29_06305	*btuB*	outer membrane receptor for transport of vitamin e and bacteriophage bf23	-0.964046	0.0233013
NF29_01740	*None*	---NA---	-0.96547	0.0029782 4
NF29_11770	*ptsH*	pts system protein hpr	-0.965508	0.0069368 5
NF29_03950	*ygaV*	hypothetical protein	-0.965678	0.0051626 8
NF29_11140	*lepB*	leader peptidase (signal peptidase i)	-0.965786	0.0034691 9
NF29_00145	*dnaE*	dna polymerase alpha subunit	-0.968797	0.0045452 1
NF29_08375	*rpmA*	50s ribosomal subunit protein l27	-0.976109	0.0059006 7
NF29_19315	*ycdY*	oxidoreductase component	-0.976696	0.003
NF29_07525	*cysG*	uroporphyrinogen iii methylase sirohaeme biosynthesis	-0.980251	0.0036752 9
NF29_00170	*lpxD*	udp-3-o-(3-hydroxymyristoyl)-glucosamine n-acyltransferase third step of endotoxin synthesis	-0.982476	0.0044660 5
NF29_18145	*ribC*	riboflavin alpha chain	-0.983709	0.0042731 9

NF29_02780	*speG*	spermidine n1-acetyltransferase	-0.985465	0.0058582 6
NF29_02365	*efp*	elongation factor p (ef-p)	-0.986518	0.0051827
NF29_05780	*rsmG*	glucose-inhibited division chromosome replication	-0.986638	0.0020970 6
NF29_05765	*atpE*	membrane-bound atp f0 subunit c	-0.989302	0.0046215 3
NF29_03580	*dinJ*	damage-inducible protein j	-0.990079	0.0017155 9
NF29_15575	*ynfK*	dethiobiotin synthetase	-0.99066	0.0035706 6
NF29_06435	*wecA*	udp- c:undecaprenylphosphate c-1-phosphate transferase synthesis of enterobacterial common antigen	-0.99087	0.0042046 2
NF29_06405	*ppiC*	peptidyl-prolyl cis-trans isomerase c (rotamase c)	-0.995114	0.0030147 2
NF29_06425	*trxA*	thioredoxin-like protein	-0.99736	0.0048243 8
NF29_19025	*acpP*	acyl carrier protein	-1.00086	0.0057232 1
NF29_01865	*ridA*	enamine/imine deaminase	-1.00474	0.0040638 1
NF29_09725	*fldB*	flavodoxin 2	-1.00837	0.0029890 1
NF29_19775	*uup*	atp-binding component of a transport system	-1.01109	0.0024303 5
NF29_08480	*rpsO*	30s ribosomal subunit protein s15	-1.01333	0.0032217 6
NF29_18465	*rplT*	50s ribosomal subunit protein and regulator	-1.01362	0.0045498 8
NF29_23010	*yafK*	transpeptidase	-1.02193	0.0030280 8
NF29_05950	*typA*	gtp-binding factor	-1.02421	0.0035844 7
NF29_08975	*None*	---NA---	-1.02474	0.0015272 3
NF29_06910	*None*	---NA---	-1.02497	0.001
NF29_05725	*glmS*	l-glutamine:d-fructose-6-phosphate aminotransferase	-1.02621	0.0023139 3
NF29_00190	*cdsA*	cdp-diglyceride synthetase	-1.03057	0.0021659 4
NF29_22310	*rcnR*	alpha helix chain	-1.03598	0.0357013
NF29_11310	*fdx*	[2Fe-2S] ferredoxin	-1.03975	0.0015818 6
NF29_00620	*mraZ*	division/cell wall cluster transcriptional repressor	-1.0399	0.0022739 9
NF29_16690	*None*	---NA---	-1.04008	0.0010423 7
NF29_17875	*None*	---NA---	-1.04088	0.0011544 6
NF29_15020	*tdk*	thymidine kinase	-1.04096	0.0021525 3
NF29_09910	fepE	ferric enterobactin transport	-1.04379	0.002

NF29_22570	*tsx*	nucleoside channel receptor of phage t6 and colicin k	-1.0448	0.000916326
NF29_08650	*tdcB*	threonine catabolic	-1.0456	0.00130322
NF29_04060	*None*	---NA---	-1.04563	0.00216594
NF29_06665	*tatA*	TatABCE protein translocation system subunit	-1.04693	0.00126124
NF29_22030	*sfmA*	fimbrial-like protein	-1.05417	0.00343729
NF29_21505	*rnk*	regulator of nucleoside diphosphate kinase	-1.05544	0.00171559
NF29_13625	*yedD*	lipoprotein	-1.05662	0.00118682
NF29_09700	*yqfB*	hypothetical protein	-1.05666	0.0067728
NF29_10700	*stpA*	dna-binding protein h-ns-like protein chaperone activity rna splicing?	-1.05771	0.00237036
NF29_13790	*yecA*	hypothetical protein	-1.0582	0.00104237
NF29_10605	*yqaA*	hypothetical protein	-1.05821	0.00631302
NF29_00225	*map*	methionine aminopeptidase	-1.05835	0.00216594
NF29_09480	*trmI*	tRNA m(7)G46 methyltransferase	-1.05859	0.0343176
NF29_05230	*gmK*	guanylate kinase	-1.06102	0.00165022
NF29_23155	*None*	---NA---	-1.06399	0.006
NF29_12230	*lrhA*	transcriptional regulator lysr-type	-1.06817	0.000626224
NF29_01220	*None*	---NA---	-1.06981	0.0012491
NF29_09605	*fbaA*	fructose-bisphosphate class ii	-1.07244	0.00252382
NF29_05695	*yieH*	6-phosphogluconate phosphatase	-1.07246	0.00056994
NF29_01785	*rraB*	regulator of ribonuclease activity B	-1.07379	0.00220734
NF29_11095	*grcA*	formate acetyltransferase	-1.07428	0.00285907
NF29_10565	*alaS*	alanyl-trna synthetase	-1.07773	0.00165022
NF29_14935	*narL*	pleiotrophic regulation of anaerobic respiration: response regulator for dms and tor genes	-1.08486	0.0295041
NF29_06215	*metJ*	repressor of all met genes but metf	-1.08615	0.00166938
NF29_08370	*rplU*	50s ribosomal subunit protein l21	-1.08893	0.00192648
NF29_00160	*lpxA*	udp-n-acetylglucosamine acetyltransferase lipid a biosynthesis	-1.09237	0.00312064
NF29_14875	*None*	---NA---	-1.1076	0.000515179
NF29_04720	*secE*	preprotein translocase	-1.10895	<0.001

NF29_04675	*chbB*	pep-dependent phosphotransferase enzyme iv for and salicin	-1.11022	0.0067279 9
NF29_01760	*None*	---NA---	-1.11153	<0.001
NF29_07250	*None*	---NA---	-1.11447	0.0029952 4
NF29_15550	*dsbC*	protein disulfide isomerase ii	-1.11629	0.0025956 2
NF29_20085	*lrp*	regulator for leucine (or lrp) regulon and high-affinity branched-chain amino acid transport system	-1.12014	0.0012995 9
NF29_15065	*yciU*	hypothetical protein	-1.12194	<0.001
NF29_04090	*hokD*	polypeptide destructive to membrane potential	-1.12361	0.0484583
NF29_09695	*alsR*	transcriptional repressor of rpib expression	-1.12412	<0.001
NF29_20145	*clpS*	ATP-dependent Clp protease adaptor	-1.12499	0.001
NF29_21475	*flc*	fluoride efflux channel, dual topology membrane protein	-1.12538	0.0017111 3
NF29_22595	*yajC*	preprotein translocase subunit	-1.12723	0.0007903 02
NF29_18805	*ymjA*	DUF2543 family protein	-1.12986	0.0074231
NF29_11975	*yfcZ*	hypothetical protein	-1.13118	0.0011251 9
NF29_00390	*hpt*	hypoxanthine phosphoribosyltransferase	-1.13126	0.0009946 71
NF29_20055	*dmsB*	fe-s subunit	-1.14131	0.0022073 4
NF29_04480	*psiE*	phosphate-starvation-inducible protein	-1.14561	0.0009521 3
NF29_08435	*secG*	protein export - membrane protein	-1.14645	0.0010423 7
NF29_17815	None	---NA---	-1.14689	<0.001
NF29_05235	*rpoZ*	rna omega subunit	-1.14784	<0.001
NF29_04790	*eptB*	KDO phosphoethanolamine transferase	-1.1491	<0.001
NF29_06115	*pfkA*	6-phosphofructokinase i	-1.15722	0.0006578
NF29_19045	*plsX*	glycerolphosphate auxotrophy in plsb background	-1.16097	<0.001
NF29_00185	*rseP*	serine endoprotease	-1.16116	<0.001
NF29_08045	*csrD*	cytochrome c-type biogenesis protein	-1.16381	<0.001
NF29_14080	*znuA*	zinc ABC transporter periplasmic binding protein	-1.16825	<0.001
NF29_22100	*None*	---NA---	-1.16869	<0.001
NF29_08405	*greA*	transcription elongation factor: cleaves 3 nucleotide of paused mrna	-1.17473	0.0005019 45
NF29_04300	*yhhY*	acyltransferase for 30s ribosomal subunit protein s18 acetylation of n-terminal alanine	-1.18122	<0.001
NF29_08410	*yhbY*	RNA-binding protein	-1.18656	<0.001
NF29_04615	*yjaG*	hypothetical protein	-1.19058	<0.001
NF29_04700	*rplJ*	50s ribosomal subunit protein l10	-1.19643	0.0005286 02

NF29_14310	*yobF*	hypothetical protein	-1.19802	0.0005020 4
NF29_13430	*mtfA*	hypothetical protein	-1.20542	<0.001
NF29_00870	*satP*	succinate-acetate/proton symporter	-1.20969	0.0009350 19
NF29_13705	*sdiA*	transcriptional regulator of ftsqaz gene cluster	-1.21014	<0.001
NF29_21400	*lptE*	a minor lipoprotein	-1.21095	<0.001
NF29_14100	*yebK*	transcriptional repressor of rpib expression	-1.21559	<0.001
NF29_04935	*malS*	trehalase 6-p hydrolase	-1.22186	<0.001
NF29_06290	*fabR*	transcriptional repressor of fabA and fabB	-1.22428	<0.001
NF29_05915	*yihA*	GTP-binding protein	-1.2302	<0.001
NF29_16530	*None*	---NA---	-1.23038	<0.001
NF29_12085	*cvpA*	membrane protein required for colicin v production	-1.23282	<0.001
NF29_12025	*yfcL*	hypothetical protein	-1.24242	<0.001
NF29_21070	*ybgE*	cyd operon protein	-1.24375	0.0357013
NF29_13710	*yecF*	hypothetical protein	-1.24965	<0.001
NF29_19750	*fabA*	beta-hydroxydecanoyl thioester trans-2-decenoyl-acp isomerase	-1.25507	<0.001
NF29_20795	*ybhQ*	hypothetical protein	-1.25803	0.0011455
NF29_07875	*smg*	hypothetical protein	-1.2584	<0.001
NF29_01140	*None*	---NA---	-1.25965	0.0008597 78
NF29_18100	*grxD*	glutaredoxin 3	-1.26009	<0.001
NF29_05175	*rpmG*	50s ribosomal subunit protein l33	-1.26134	<0.001
NF29_19640	*None*	---NA---	-1.26289	<0.001
NF29_05890	*yihD*	hypothetical protein	-1.26785	<0.001
NF29_12555	*mepS*	putative lipoprotein	-1.27287	<0.001
NF29_15405	*None*	---NA---	-1.27745	<0.001
NF29_09970	*rppH*	invasion protein	-1.2785	<0.001
NF29_06915	*pitA*	low-affinity phosphate transport	-1.27882	<0.001
NF29_02170	*yjfO*	biofilm peroxide resistance protein	-1.27892	<0.001
NF29_17005	*lamB*	phage lambda receptor protein maltose high-affinity receptor	-1.28084	0.0016288
NF29_08490	*nlpI*	control proteins	-1.29203	<0.001
NF29_05775	*atpI*	membrane-bound atp dispensable affects expression of atpb	-1.29564	<0.001
NF29_13275	*yeeX*	alpha helix protein	-1.29723	<0.001
NF29_14385	*yoaB*	hypothetical protein	-1.30072	<0.001
NF29_11845	yfeD	hypothetical protein	-1.30397	<0.001
NF29_13715	*uvrY*	2-component transcriptional regulator	-1.30932	<0.001
NF29_15230	*None*	---NA---	-1.31211	0.0007076 86

NF29_21075	*None*	---NA---	-1.31838	0.0110527
NF29_11850	*yfeC*	hypothetical protein	-1.33862	<0.001
NF29_21080	*cydB*	cytochrome d terminal oxidase polypeptide subunit ii	-1.3513	<0.001
NF29_14315	*cspC*	cold shock protein	-1.35378	<0.001
NF29_05185	*yicR*	dna repair protein	-1.36388	<0.001
NF29_18180	*None*	---NA---	-1.36657	0.0035844 7
NF29_19030	*fabG*	5-keto-d-gluconate 5-reductase	-1.38488	<0.001
NF29_00165	*fabZ*	(3R)-hydroxymyristol acyl carrier protein dehydratase	-1.39148	<0.001
NF29_13815	*None*	---NA---	-1.39554	0.0047005 4
NF29_18110	*sodB*	superoxide iron	-1.40815	<0.001
NF29_07580	*yhfA*	hypothetical protein	-1.41363	<0.001
NF29_15455	*None*	---NA---	-1.41505	<0.001
NF29_22600	*tgt*	trna-guanine transglycosylase	-1.41742	<0.001
NF29_08850	*yqjI*	transcriptional regulator	-1.42051	<0.001
NF29_07985	*fis*	site-specific dna inversion stimulation factor dna-binding protein a trans activator for transcription	-1.42109	<0.001
NF29_11810	*None*	---NA---	-1.42139	0.0022073 4
NF29_05180	*rpmB*	50s ribosomal subunit protein l28	-1.43677	<0.001
NF29_07535	*nirB*	nitrite reductase (nad h) subunit	-1.43937	<0.001
NF29_11115	*rpoE*	rna sigma-e factor heat shock and oxidative stress	-1.45532	<0.001
NF29_09595	*epd*	d-erythrose 4-phosphate dehydrogenase	-1.45729	<0.001
NF29_05060	*gpmM*	putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	-1.45801	<0.001
NF29_09645	*zapA*	cell division protein	-1.46004	<0.001
NF29_01825	*tabA*	biofilm modulator regulated by toxins	-1.48786	<0.001
NF29_18040	*slyA*	transcriptional regulator for cryptic hemolysin	-1.49109	<0.001
NF29_15165	*None*	---NA---	-1.49178	<0.001
NF29_13875	*flhD*	regulator of flagellar acting on class 2 operons transcriptional initiation factor	-1.49973	<0.001
NF29_17175	*fdnG*	formate dehydrogenase- nitrate- alpha subunit	-1.50032	0.0010423 7
NF29_09000	*rpsU*	30s ribosomal subunit protein s21	-1.50689	<0.001
NF29_04850	*cspA*	cold shock protein transcriptional activator of hns	-1.51208	<0.001
NF29_06135	*tpiA*	triosephosphate isomerase	-1.51604	<0.001
NF29_20675	*None*	---NA---	-1.52333	<0.001
NF29_13820	*ftnA*	cytoplasmic ferritin (an iron storage protein)	-1.52374	<0.001
NF29_07990	*dusB*	regulator protein	-1.52866	<0.001

NF29_19055	*yceD*	hypothetical protein	-1.53357	<0.001
NF29_22630	*malZ*	maltodextrin glucosidase	-1.58799	<0.001
NF29_05520	*dsdC*	transcriptional regulator lysr-type	-1.59098	<0.001
NF29_12505	*rplY*	50s ribosomal subunit protein l25	-1.59106	<0.001
NF29_17190	*nmpC*	outer membrane porin protein locus of qsr prophage	-1.60239	<0.001
NF29_21515	*uspG*	filament protein	-1.61184	<0.001
NF29_11225	*glnB*	regulatory protein p-ii for glutamine synthetase	-1.62296	<0.001
NF29_21040	*pal*	peptidoglycan-associated lipoprotein	-1.62954	<0.001
NF29_08660	*tdcD*	acetate kinase	-1.63886	<0.001
NF29_04525	*yjbD*	DUF3811 family protein	-1.64397	<0.001
NF29_07530	nirD	nitrite reductase (nad h) subunit	-1.68441	0.007
NF29_11970	*fadL*	transport of long-chain fatty acids sensitivity to phage t2	-1.69753	<0.001
NF29_02485	*yjdM*	zinc-ribbon family protein	-1.74052	<0.001
NF29_13880	*flhC*	regulator of flagellar biosynthesis acting on class 2 operons transcription initiation factor	-1.75895	<0.001
NF29_21045	*tolB*	periplasmic protein involved in the tonb-independent uptake of group a colicins	-1.81124	<0.001
NF29_07350	*malP*	maltodextrin phosphorylase	-2.25258	<0.001
NF29_17170	*fdnH*	formate dehydrogenase- nitrate- iron-sulfur beta subunit	-2.39407	0.0115434
NF29_04450	*malM*	periplasmic protein of mal regulon	-2.70674	<0.001
NF29_17180	*fdnG*	formate dehydrogenase- nitrate- alpha subunit	-2.76031	<0.001
NF29_07355	*malQ*	4-alpha-glucanotransferase (amylomaltase)	-2.82673	<0.001
NF29_04470	*malF*	part of maltose periplasmic	-2.83987	<0.001
NF29_13650	*fliC*	flagellar biosynthesis filament structural protein	-2.91338	<0.001
NF29_05530	*dsdA*	d-serine dehydratase	-3.08263	<0.001
NF29_04475	*malG*	part of maltose inner membrane	-3.42657	<0.001
NF29_04460	*malK*	atp-binding component of a transport system	-3.52376	<0.001
NF29_17165	*fdnI*	formate dehydrogenase- nitrate- cytochrome b556 gamma subunit	-3.52976	<0.001
NF29_05525	*dsdX*	transport system permease	-3.72761	<0.001

Supplementary Table 3. PhoQ regulated genes identified from RNAseq analysis. After RNAseq analysis of R/S subpopulations, genes that were differentially expressed between the populations.

Locus	Gene Homolog	Predicted Function	Fold Change After Induction (Log2)	q value	Ref.
NF29_17785	*acrB*	acridine efflux pump	3.4121	<0.001	[18]
NF29_18075	*eptA/pagB/pmrC*	lipid A phosphoethanolamine transferase	2.60527	<0.001	[19]
NF29_16360	*yghA*	2-deoxy-d-gluconate 3-dehydrogenase	2.55599	<0.001	[20]
NF29_20485	*ybjG*	undecaprenyl pyrophosphate phosphatase	2.45085	<0.001	[18,20]
NF29_20990	*ybgS*	homeobox protein	2.41473	<0.001	[20]
NF29_07125	*arnB/pmrH*	aminotransferase, PLP-dependent	2.41056	<0.001	[21,22]
NF29_11685	*tktB*	transketolase 2 isozyme	2.14843	<0.001	[23]
NF29_04505	*yjbE*	extracellular polysaccharide production threonine-rich protein	1.99874	<0.001	[20]
NF29_11690	*talB*	transaldolase a	1.83033	<0.001	[23]
NF29_10510	*srlD*	glucitol -6-phosphate dehydrogenase	1.58928	0.023	[18]
NF29_21110	*sucC*	succinyl- beta subunit	1.4085	0.007	[18]
NF29_17110	*adhP*	alcohol dehydrogenase	1.25113	<0.001	[20]
NF29_00805	*carA*	carbamoyl phosphate synthase small subunit CDS	1.09651	<0.001	[18]
NF29_21115	*sucB*	2-oxoglutarate dehydrogenase (dihydrolipoyltranssuccinase e2 component)	1.09252	0.001	[18]
NF29_20670	*dps*	global starvation conditions	1.07932	0.002	[20]
NF29_06185	*hslU*	heat shock protein atpase homologous to chaperones	1.07091	<0.001	[20]
NF29_12260	*nuoG*	nadh dehydrogenase i chain g	1.03827	0.010	[18]
NF29_21870	*mgtA*	mg2+ transport p-type 1	0.909424	0.004	[20,24]
NF29_15365	*rstA*	response transcriptional regulatory protein (sensor)	0.899441	0.040	[18,20,25]
NF29_07390	*feoB*	ferrous iron transport protein b	0.884113	0.007	[18,25]
NF29_01855	*pyrB*	aspartate catalytic subunit	0.865969	0.010	[18]
NF29_22255	*acrA*	membrane protein	0.803824	0.025	[18]
NF29_12760	*dld*	d-lactate fad nadh independent	0.780696	0.016	[20]
NF29_01280	*cstA*	carbon starvation protein	0.772438	0.028	[20]
NF29_14230	*virK/ybjX*	putative enzyme	0.729342	0.034	[18,20]
NF29_16290	*yhjC*	transcriptional regulator lysr-type	-0.784109	0.021	[18]

NF29_09675	*gcvH*	in glycine cleavage carrier of aminomethyl moiety via covalently bound lipoyl cofactor	-0.796513	0.031	[23]
NF29_08500	*mtr*	tryptophan-specific transport protein	-0.810735	0.020	[20]
NF29_02310	*frdA*	fumarate flavoprotein subunit	-0.830201	0.029	[18]
NF29_11305	*hscA*	heat shock member of hsp70 protein family	-0.876531	0.007	[18]
NF29_20010	*serC*	3-phosphoserine aminotransferase	-0.883788	0.012	[18]
NF29_04510	*pgi*	glucosephosphate isomerase	-0.892992	0.013	[20]
NF29_02780	*speG*	spermidine n1-acetyltransferase	-0.985465	0.006	[18]
NF29_20055	*dmsB*	fe-s subunit	-1.14131	0.002	[18]
NF29_06115	*pfkA*	6-phosphofructokinase i	-1.15722	0.001	[18]
NF29_04935	*malS*	trehalase 6-p hydrolase	-1.22186	<0.001	[18]
NF29_07985	*fis*	site-specific dna inversion stimulation factor dna-binding protein a trans activator for transcription	-1.42109	<0.001	[20]
NF29_07535	*nirB*	nitrite reductase (nad h) subunit	-1.43937	<0.001	[18]
NF29_18040	*slyA*	transcriptional regulator for cryptic hemolysin	-1.49109	<0.001	[18]
NF29_17190	*nmpC*	outer membrane porin protein locus of qsr prophage	-1.60239	<0.001	[20]
NF29_08660	*tdcD*	acetate kinase	-1.63886	<0.001	[20,24]
NF29_11970	*fadL*	transport of long-chain fatty acids sensitivity to phage t2	-1.69753	<0.001	[18,20]
NF29_04450	*malM*	periplasmic protein of mal regulon	-2.70674	<0.001	[18]
NF29_04470	*malF*	part of maltose periplasmic	-2.83987	<0.001	[18,23]
NF29_04475	*malG*	part of maltose inner membrane	-3.42657	<0.001	[18,23]
NF29_04460	*malK*	atp-binding component of a transport system	-3.52376	<0.001	[18]
NF29_05525	*dsdX*	transport system permease	-3.72761	<0.001	[20]
NF29_04455	*lamB*	phage lambda receptor protein maltose high-affinity receptor	-3.80665	<0.001	[18]

Supplementary Table 4. Primers Used for Bacterial Cloning.

Primer Name	Sequence (5'-3')	Application
110	CAACAGGTTGAACTGCTGATCTTCGCCTTTTTACGGTTCCTGGCC	linearize pEX100T
111	GGTTTAACGGTTGTGGACAACAAGTTTCTACGGGGTCTGACGCTC	linearize pEX100T
108	GGCCAGGAACCGTAAAAAGGCGAAGATCAGCAGTTCAACCTGTTG	R6K ori
109	GAGCGTCAGACCCCGTAGAAACTTGTTGTCCACAACCGTTAAACC	R6K ori
142	TATGATAGAATTTGACGTCGCCCCGGTTTACTCAATGTTTATCC	phoQ promoter region
143	GTATGTGGCGTAAAATCCCTCTCATTTACAGGTGTTCATTGAGATAATAATC	phoQ promoter region
144	GATTATTATCTCAATGAACACCTGTAAATGAGAGGGATTTTACGCCACATAC	phoQ gene
145	GGTCTGCTAGTTGAACGGATCTTAACTATCGTTCAATGTGGGCTGC	phoQ gene
146	GCCCACATTGAACGATAGTTAAGATCCGTTCAACTAGCAGACCATTATC	linearize pBAV-1K-T5-GFP
147	CATTGAGTAAACCGGGGCGACGTCAAATTCTATCATAATTGTGGTTTC	linearize pBAV-1K-T5-GFP
79	GAATTACGCTAATTGAAAATTTTTTTTTTGACTCAATATCTAGACTTGC	HmR cassette
80	CCTCGCATTTTTCACATAACGGGTCAGGCGCCGGGGGCGGTG	HmR cassette
81	GAGTCAAAAAAAAAATTTTCAATTAGCGTAATTCGAACAGGTAGCCC	phoQ upstream fragment
118	CTCATTACCCTGTTATCCCTACCCGGGCGCATTGCTACGTCATCACCTG	phoQ upstream fragment
82	ACCGCCCCCGGCGCCTGACCCGTTATGTGAAAAATGCGAGG	phoQ downstream fragment
119	GCTCTAGGGATAACAGGGTAATCCCGGGAATATCACCCGGCTCCAGC	phoQ downstream fragment
eptA-1F	GCCATTATTGCCCCTTCGCA	qRT-PCR of eptA
eptA-1R	AGCGCATCCGATCGTCAAT	qRT-PCR of eptA
arnB-2F	CGCCGGAACGTACTACAAGA	qRT-PCR of arnB
arnB-2R	GGGCATTATCCGTGACGACT	qRT-PCR of arnB
rpoD-F3	TGCGAAGAAAGAGATGGTTG	qRT-PCR of rpoD
rpoD-R3	GGTGGAGAACTTGTAACCAC	qRT-PCR of rpoD

CHAPTER 3: Resistance Inhibitor Suppresses Lipid A Modifications and Reverses Colistin Resistance in *Acinetobacter baumannii*

Note: Chapter 3 is adapted from a manuscript in collaboration with Yuhong Du, Spandan Chennamadhavuni, Chui Yoke Chin, Jordan Valdez, Dennis Liotta, Haian Fu, James Snyder, James Nettles, and David Weiss from Emory University along with Courtney Chandler and Robert Ernst from the University of Maryland, Baltimore

Author Contributions: DSW and EKC designed the study. EKC, YD, and HF performed the high throughput in vitro screen. JN and JS performed the *in silico* screen. SC and DL synthesized compounds and performed chemical analyses, EKC wrote the manuscript

Abstract

Antibiotic resistant bacterial infections are a significant cause of morbidity and mortality in the United States, accounting for greater than 2 million cases and 23,000 deaths annually. If left unchecked, deaths due to antibiotic resistance are predicted to reach 10 million each year, roughly equivalent to one death every 3 seconds. According to the World Health Organization, multidrug resistant infections caused by the Gram negative nosocomial pathogen *Acinetobacter baumannii* are a critical concern, resulting in treatment with last-line antibiotics such as the outer membrane-targeting cationic antimicrobial colistin. Colistin resistance in *A. baumannii* is controlled by the NaxD deacetylase, a protein involved in modification of the outer membrane lipid A component of lipopolysaccharide (LPS). A small molecule inhibitor of NaxD was identified by two tandem high-throughput screens: (1) an *in vitro* cell-based screen for molecules that re-sensitize resistant bacteria to colistin and (2) an *in silico* screen for compounds predicted to bind NaxD using a protein homology model. The inhibitor restored bactericidal activity of colistin against colistin-resistant *Acinetobacter baumannii.* Inhibitor mediated killing was due to a *naxD*-dependent decrease in the colistin minimal inhibitory concentration, with no effect on bacterial viability in the absence of antibiotic. Furthermore, mass spectrometry revealed a reduction in colistin-resistant lipid A modifications in the presence of inhibitor. Finally, the inhibitor reduced the colistin minimal inhibitory concentration of *A. baumannii* up to 128-fold, restoring colistin susceptibility in highly resistant clinical strains. This small-molecule inhibitor demonstrates that modulation of an outer membrane resistance response can re-sensitize *A. baumannii* to the last-line antibiotic colistin.

Introduction

In a 2013 report released by the CDC, it was estimated that greater than 23 million antibiotic resistant infections occur annually in the United States, accounting for at least 23,000 deaths each year and placing a significant strain on the healthcare system (1). The burden of antibiotic resistance is not restricted to the United States, as a separate report sponsored by the Wellcome Trust and the UK Department of Health estimated that nearly 700,000 deaths were attributed to antibiotic resistance worldwide in 2014 (2). Antibiotic resistant infections caused by carbapenem-resistant *Acinetobacter baumannii* are of particular concern, as this organism was recently named a critical priority pathogen by the World Health Organization for which new antibiotics need to be developed (3). The incidence of carbapenem-resistant *A. baumannii* infections has increased in recent years, with reports of resistance commonly ranging between 45-64% of all isolates observed (4-7). As a result, treatment with polymyxin antibiotics such as colistin in either monotherapy or in combination with other drugs is sometimes the only therapeutic option (8-10). Unfortunately, reports of colistin resistance in carbapenem-resistant *A. baumannii* infections have already surfaced (7, 11).

Colistin is a bactericidal cationic antimicrobial peptide antibiotic that exerts its activity through interactions with the negatively charged lipid A molecule in the outer membrane of Gram negative bacteria (12, 13). Upon association with lipid A, colistin subsequently disrupts the outer membrane to induce membrane depolarization and leakage of contents (14). Resistance to the cationic antibiotic colistin in *A. baumannii* is typically associated with decoration of the phosphate groups on lipid A by molecules such as phosphoethanolamine that mask the phosphate-associated negative charge, thereby reducing polymyxin affinity (15, 16). Our laboratory recently identified *naxD* as a gene that supports polymyxin resistance in *A. baumannii* by facilitating the addition of

galactosamine to the outer membrane molecule lipid A (17). NaxD is a membrane-associated deacetylase of the YdjC superfamily that converts undecaprenyl phosphate (UDP) linked N-acetylgalactosamine to galactosamine, a precursor step that was previously found to be required for galactosamine attachment to the terminal phosphate on lipid A (17, 18). Furthermore, *naxD* mutant strains demonstrated reduced survival in the presence of polymyxin, indicating that inhibition of this protein could prove a promising target for reversing colistin resistance in *A. baumannii* and restoring susceptibility to this last-line drug (17).

In this work, we present a strategy to inhibit lipid A modification by galactosamine and reduce colistin resistance in *A. baumannii* through the inhibition of NaxD with a small molecule inhibitor. Two tandem screens were used to identify compounds with (1) high predicted affinity for the binding pocket of a NaxD using an *in silico* homology model and (2) that synergize with colistin to inhibit the growth of colistin resistant *Acinetobacter*. Hits were assessed for conservation of a core chemotype, then further analyzed for *naxD*-dependent reduction in colistin resistance and inhibition of lipid A modifications. Finally, we demonstrate that a *naxD*-dependent small molecule inhibitor increases colistin susceptibility in an assortment of colistin resistant clinical strains, highlighting the potential for resistance inhibitor antibiotic adjuvants as a strategy to combat resistance to last-line antibiotics in multi-drug resistant *A. baumannii* infections.

Results

Tandem screens identify a conserved chemotype that promotes colistin-dependent growth inhibition. Two separate screens were utilized to identify small molecule compounds that inhibit *A. baumannii* growth only in the presence of colistin and also have high affinity for the NaxD colistin resistance protein. The first screen was performed *in vitro* using the previously reported laboratory-adapted colistin resistant *A. baumannii* strain MAC204 that has a colistin minimal inhibitory concentration (MIC) of 16 μg/mL (19) (**Figure 1 A).** Bacterial growth inhibition was assessed for 133,920 compounds in a high throughput screen using 40 μM concentrations of each compound with the addition of 1μg/mL colistin. From this screen, 622 compounds demonstrated greater than 50% growth inhibition of MAC204 relative to an untreated control (0.46% hit rate) **(Figure 1B)**. Of those primary hits, 56 compounds (0.042% total hit rate) exerted at least 3-fold more potent inhibition in when tested in combination with colistin relative to a compound only control. The top hit molecule from this screen, designated "SC030", inhibited growth of MAC204 by greater than 90% when applied in combination with colistin but demonstrated less than 10% inhibition when used as a standalone treatment (**Figure 1B)**. Screen 2 was performed *in silico* by docking 728,000 compound structures from an assortment of drug discovery chemical libraries into the binding pocket of a NaxD homology model, built upon the YdjC family protein crystal structure from *Thermus thermophiles* (**Figure 1A)** (20). From this *in silico* screen, the top 34 compounds with highest computed binding affinities for NaxD were selected for *in vitro* analysis of colistin-dependent growth inhibition, as in screen 1. Of the top 34 compounds identified from screen 2, a single compound designated "SC021" demonstrated greater than 40% growth inhibition in the presence of colistin while exerting less than 10% growth inhibition as a single treatment (**Figure 1C).** Structural analysis of the top compound from each

screen revealed a conserved chemotype associated with colistin-dependent growth inhibition (**Table 1, blue)** that consisted of a carboxamide linked thiazole ring with variation in functional groups at each end of the molecule. The molecular structure of the screen 1 compound SC030 consisted of a 5-nitrofuran group attached to a 4.5-dichloro benzothiazole group via an amide bond, while the screen 2 hit SC021consisted of a 3-thio-4H-1,2,4-triazole group attached to 4,5-dihydro naphtho thiazole group, also via an amide bond (**Table 1**). Finally, we docked the compound structure from screen 1, which contained the most favorable inhibition activities in the presence and absence of colistin, into the NaxD homology model binding pocket to identify how this compound could be interacting with the protein in relation to the native substrate UDP-N-acetylgalactosamine. Docking revealed that both the inhibitor and the native substrate attain predicted interactions with the putative NaxD catalytic triad at amino acids Asp 10, His 61, and His 119 (**Figure 1E**). Surprisingly, the inhibitor and native substrate appeared to inhabit completely distinct recognition grooves within the NaxD model, suggesting that additional chemotypes could potentially confer NaxD inhibition through an alternative catalytic site access channel (**Figure 1E)**.

Inhibitor restores colistin susceptibility through *naxD*. After identification of a conserved chemotype that promoted colistin-dependent growth inhibition of *A. baumannii*, we next wanted to assess the degree of growth inhibition and also confirm that activity was dependent on *naxD.* To do so, we decided to focus on inhibitor SC030 identified in screen 1, which conferred the strongest desired activities from all molecules tested of (1) high growth inhibition in the presence of colistin combined with (2) low inhibition as a standalone treatment (**Figure 1**). To confirm the *in vitro* screen assay results and determine if the inhibitor in combination with colistin was bactericidal or simply prevented growth of colistin resistant *A. baumannii*, bacterial growth

was assessed over time during treatment with either the inhibitor or the inhibitor plus colistin. In control conditions, colistin resistant MAC204 cultures inoculated with 10^5 CFU/mL grew to a final density of approximately $4x10^8$ CFU/mL after 8 hours of growth, and supplementation with neither 1 μg/mL colistin alone nor 40 μM inhibitor affected final density of the culture. This confirmed that MAC204 was indeed resistant to 1 μg/mL colistin and that the inhibitor exhibited no growth-inhibitory activity over time (**Figure 2A**). Growth media supplementation with inhibitor-colistin combination again prevented outgrowth of MAC204 as previously determined in screen 1, and CFU decreased to below the limit of detection within 2 hours post-inoculation, indicating that the inhibitor restored bactericidal activity of colistin against the colistin resistant MAC204. Next, the MIC of colistin in the presence or absence of 40μM inhibitor was assessed to determine if the inhibitor could reduce colistin resistance to a clinically susceptible level of less than 4 μg/mL by broth microdilution assay. Broth microdilution confirmed that MAC204 was resistant to colistin in the absence of inhibitor, with an MIC ranging between 16-32 μg/mL (**Figure 2B)**. Addition of 40 μM inhibitor conferred a colistin MIC reduction to 2 μg/mL, indicating that the inhibitor could restore clinical susceptibility in a colistin resistant strain (**Figure 2B**). In order to determine if inhibitor activity was *naxD* dependent, colistin MIC was evaluated in both the resistant wild type and a Δ*naxD* mutant strain. Elevated colistin resistance was previously found to require NaxD (17), therefore the colistin MIC of the mutant strain should be susceptible and the inhibitor should not further alter colistin MIC unless acting on a different cellular pathway. As previously described, colistin MIC of MAC204Δ*naxD* was within the range of clinical susceptibility at 2 μg/mL (**Figure 2B).** Furthermore, addition of the inhibitor had little effect on the colistin MIC of the *naxD* mutant, indicating that the inhibitor indeed targets colistin resistance specifically via *naxD* **(Figure 2B**). Finally, we assessed the potency of colistin resistance inhibition to determine

if the desired activity could be achieved with nanomolar concentrations of SC030, as is typically preferred for drug candidates identified through primary screens (21). Potency of inhibition was tested using a sublethal 0.1X MIC colistin concentration for either wild type MAC204 or Δ*naxD* strains, with the addition of varying concentrations of inhibitor to determine potency. As before, the inhibitor exhibited little effect on growth of the *naxD* mutant in the presence of sublethal concentrations of colistin, however a 500 nM concentration of inhibitor SC030 completely restored colistin susceptibility to the resistant wild type strain, with an IC50 of 179 nM (**Figure 2C).** This nanomolar IC50 indicates that the inhibitor has strong activity at the low doses favorable for compounds of therapeutic value.

Inhibitor blocks lipid A modifications on cellular surface. Colistin resistant strains of *A. baumannii* are reported to express phosphoethanolamine and galactosamine (GalN) modifications on the outer membrane molecule lipid A (17, 19). Furthermore, GalN modification was found to be dependent on *naxD* in *A. baumannii* (17). Given that activity of the colistin resistance inhibitor required the presence of *naxD*, we next wanted to confirm that the inhibitor also blocks later stages of the resistance pathway that occur via GalN modification to lipid A. To do so, we grew cultures of colistin resistant MAC204 in control conditions or with the addition of 40μM inhibitor SC030 and isolated the lipid A fraction from cells. Lipid A was then analyzed by MALDI-TOF mass spectrometry to determine the presence or absence of GalN modification, as previously described (17). Under control conditions in the absence of inhibitor, mass spectrometry revealed primary peaks corresponding to bis-phosphorylated hepta-acylated lipid A (*m/z* = 1910) and that modified to contain either phosphoethanolamine (*m/z* = 2033), galactosamine (*m/z* = 2071), or a potential double galactosamine modification (*m/z* = 2216) (**Figure 3A)**. After the addition of inhibitor, the 1910 *m/z* peak corresponding to unmodified lipid A remained relatively

unchanged, however there was substantial reduction in the 2033 *m/z* peak representing phosphoethanolamine modification and almost complete absence of peaks 2071 *m/z* and 2216 *m/z* corresponding to single and double galactosamine modification, respectively (**Figure 3B**). Altogether, these results indicate that the inhibitor blocks critical lipid A modifications on the cellular surface to restore colistin susceptibility in a resistant strain.

NaxD Inhibitor restores colistin susceptibility in highly resistant clinical strains. Despite the promising activity of inhibitor SC030 to block colistin resistance in preliminary screens using a laboratory derived colistin-resistant strain of *A. baumannii*, we were still uncertain if SC030 could work effectively against other strains of *A. baumannii,* particularly clinical strains. Clinical isolates are known to utilize a variety of pathways to generate antibiotic resistance during infections, as environmental factors that help drive evolution of resistance may vary (22). Therefore, we next wanted to determine if the inhibitor could also reduce colistin resistance in *A. baumannii* strains isolated from clinical infections. To do so, colistin MIC was determined with and without the inhibitor for a panel of colistin resistant *A. baumannii* clinical isolates representing a variety of infection types and geographic locations. For all strains tested, colistin MIC under control conditions in the absence of inhibitor ranged between 8 μg/mL to 256 μg/mL, indicating that all strains tested had elevated colistin MIC and could be clinically classified as resistant (**Figure 4**). We then tested colistin resistance with the addition of 800 nM inhibitor, a concentration slightly higher than the IC50 determined using the laboratory strain MAC204 but still within the nanomolar range preferred for activity screens. MAC204 was tested as a control with these adjusted conditions, and as previously demonstrated (**Figure 2B)**, addition of the inhibitor reduced the colistin MIC of MAC204 from a clinically resistant level down to 1 μg/mL, equivalent to an approximate 8-fold decrease in resistance (**Figure 4)**. Likewise, addition of the inhibitor reduced

colistin MIC by at least 8-fold in 7 of the 8 clinical strains tested (**Figure 4).** Notably, the inhibitor was able to restore colistin susceptibility and reduce colistin MICs from 256 μg/mL to less than 2 μg/mL in even the highly resistant strains CI-4 and ARCL, representing an approximate 128-fold decrease in colistin MIC. These results highlight the promising potential of compound SC030 to exhibit broad colistin resistance inhibition against *A. baumannii* strains of clinical origin of from a variety of backgrounds.

Discussion

The development of colistin resistance in carbapenem *A. baumannii* infections is of critical concern, as colistin is one of the only remaining treatment options for such infections (8-10). As a novel strategy to combat colistin resistance, we sought to develop a small molecule inhibitor that would restore colistin susceptibility in *A. baumannii* strains that were already colistin resistant while exerting negligible effects on bacterial viability when given alone, thereby rescuing the efficacy of colistin as a last resort drug. Such strategies have been successfully implemented for some beta-lactam antibiotics with the development of clavulanic acid as an inhibitor of beta-lactamase resistance enzymes (23). Despite the success of clavulanic acid, relatively few studies have evaluated such antibiotic adjuvants in the context of colistin resistance, and no studies to date focus on inhibiting galactosamine modification of lipid A (24, 25).

Through tandem *in silico* and *in vitro* screens, we successfully identified a small molecule inhibitor chemotype of colistin resistance in *A. baumannii*. The top candidate molecule identified in the screens, SC030, exhibited little effect on bacterial growth when administered alone, however was able to restore colistin susceptibility in a colistin resistant strain. Inhibition of colistin resistance was dependent on the *naxD* gene that promotes GalN modification of lipid A in *A. baumannii,* and the inhibitor effectively blocked this lipid A modification. Finally, the colistin resistance inhibitor was effective in the majority of clinical strains tested, indicating that inhibitor SC030 has the potential to act on *A. baumannii* strains of clinical relevance. It is currently unknown why SC030 displayed little effect in one of the strains tested, however recent studies suggest that a poorly characterized mechanism of colistin resistance that is independent of lipid A modification can occur in *Acinetobacter,* and requires complete loss of lipid A from the outer membrane (26). This unique resistance mechanism is likely independent of *naxD* and could explain the lack of

inhibitor effect, however the relevance and prevalence of this mechanism is unknown. Ultimately, inhibitor SC030 was capable of restoring colistin susceptibility in highly resistant clinical strains of *A. baumannii* that had presenting colistin MICs equal to 256 μg/mL. Taken together, these results highlight the potential for small molecule antibiotic adjuvants to restore susceptibility to the last resort antibiotic colistin, and paves the way for a new strategy to reduce the impacts of colistin resistance in *A. baumannii* by modulating expression of resistance modifications to the colistin target lipid A.

Acknowledgements

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Figures

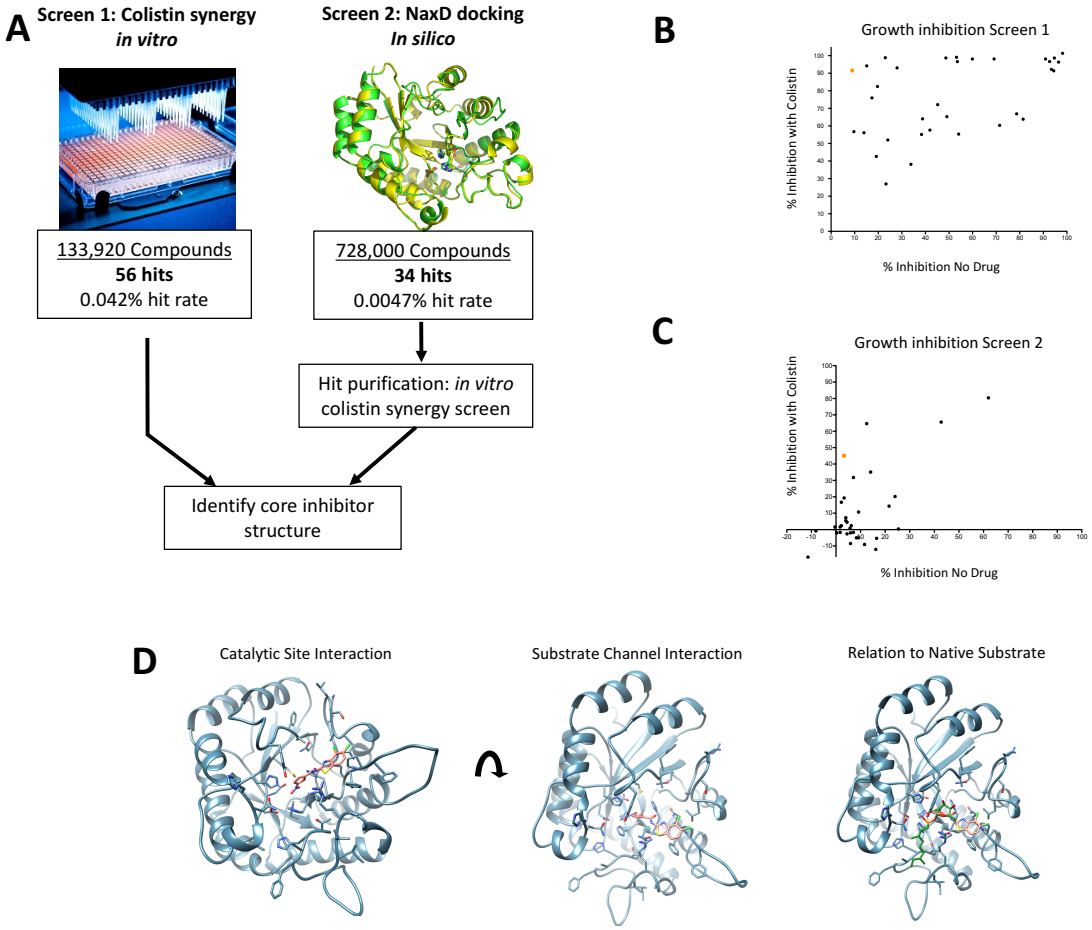


Figure 1. **Tandem screens to identify small molecule NaxD inhibitors that synergize with colistin. (A)** Overall screen strategy. Compounds were screened either for *in vitro* for colistin-dependent growth inhibition of a colistin resistant *A. baumannii* strain (screen 1) or high affinity binding *in silico* to the NaxD-homology model based on the YdjC crystal structure from *Thermus thermophilus* (screen 2). **(B)** Colistin-dependent growth inhibition for top 34 primary hits from *in vitro* screen 1, with the final top hit compound displayed in orange. **(C)** Colistin-dependent growth inhibition from *in vitro* synergy testing for top 34 hits from screen 2, with the top hit compound displayed in orange. **(D)** Docking of screen 1 top hit "SC030" (in salmon) into *Acinetobacter baumannii* NaxD homology model (in blue), showing proposed interaction with catalytic site

(left), substrate channel (middle), and in relation to the functional portion of the native substrate (in green) (right).

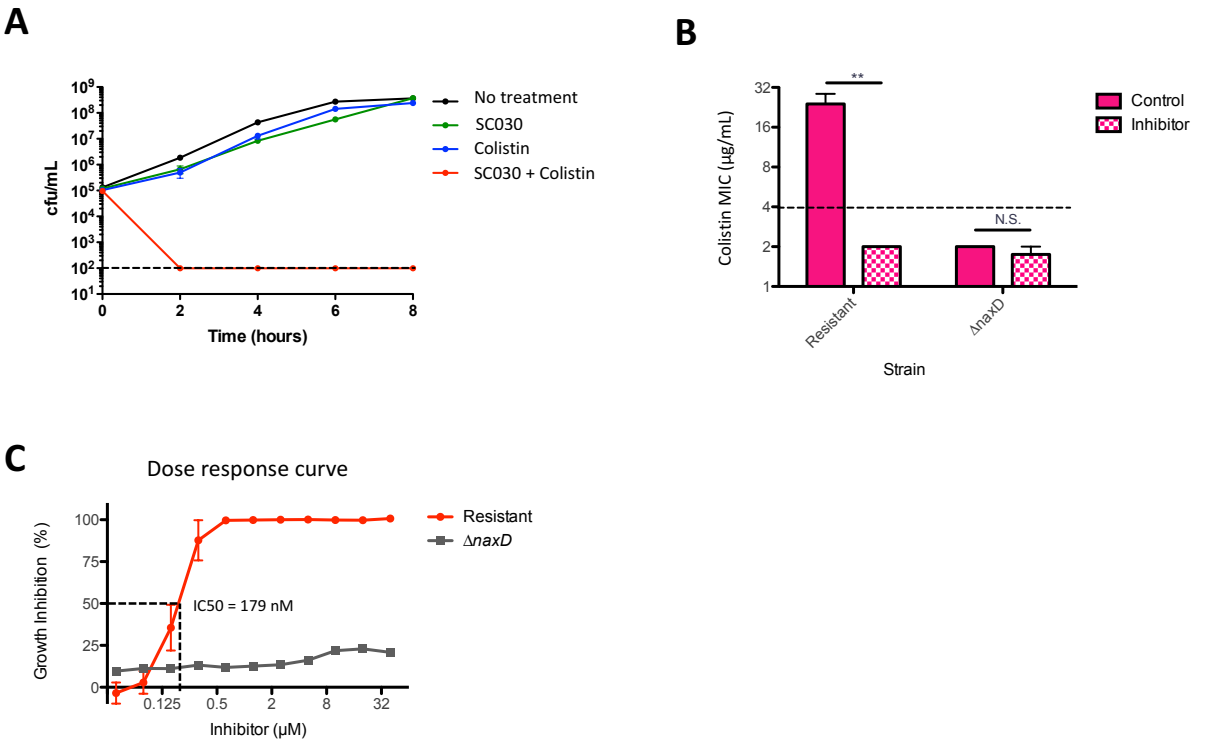


Figure 2. SC030 restores colistin susceptibility through NaxD. **(A)** Killing assays were performed on wild type MAC204 using either 40µM inhibitor SC030 (green), 1µg/mL colistin (blue), or 1µg/mL colistin plus 40µM inhibitor (red), relative to a no treatment control (black). Assay limit of detection indicated by dashed line. **(B)** Broth microdilution assessment of colistin MIC was performed with colistin alone (solid bars) or with the addition of 40µM inhibitor SC030 (hatched bars). $P<0.05$ by t-test. Breakpoint for clinical susceptibility indicated by dashed line. **(C)** Dose response curves for growth inhibition of wild type colistin resistant MAC204 and ΔnaxD strains was performed using a 0.1X MIC sublethal concentration of colistin for each strain with varying inhibitor doses. IC50=179nM, determined using GraphPad Prism v. 5.0.

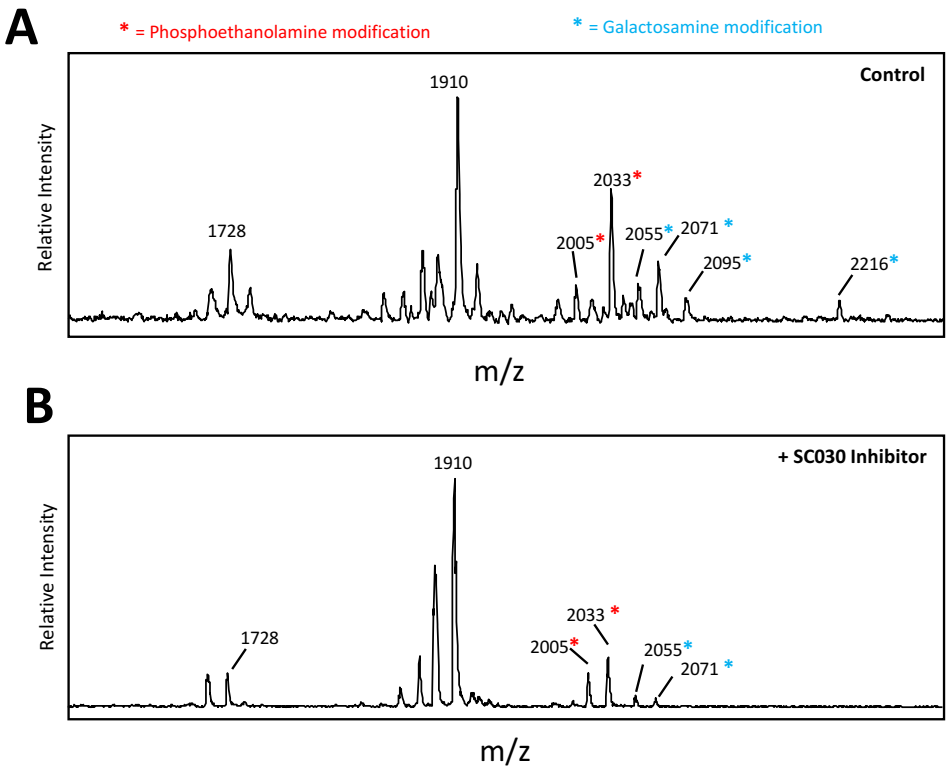


Figure 3. SC030 inhibits colistin resistant lipid A modifications. (A) MALDI-TOF profile of lipid A harvested from colistin resistant MAC204 grown under control conditions. (**B**) MALDI-TOF profile of lipid A harvested from MAC204 grown with 40μM inhibitor SC030. Modifications that correspond to phosphoethanolamine are highlighted by a red asterisk, while those representing galactosamine modification are highlighted by a blue asterisk.

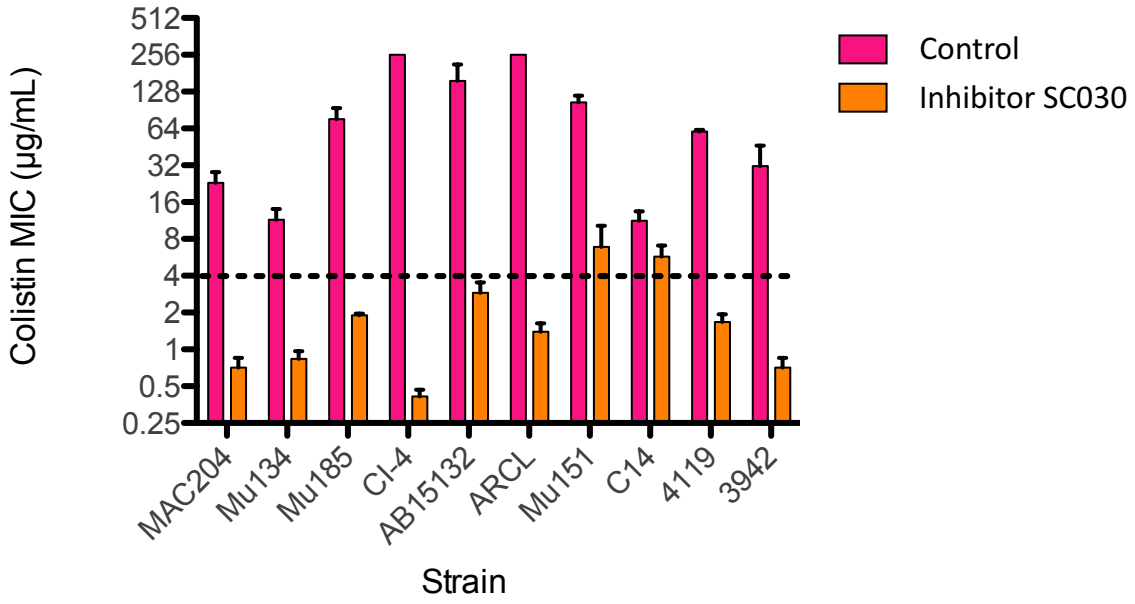


Figure 4. **Colistin MIC reduced in highly resistant clinical strains.** Colistin MIC was assessed by broth microdilution under control conditions (pink bars) or with the addition of 800nM inhibitor SC030 (orange bars). Strain MAC204 was utilized as a control. Breakpoint for clinical susceptibility indicated by dashed line.

Tables

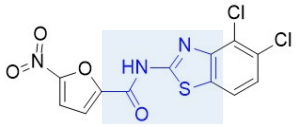
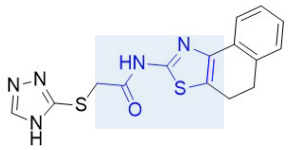
Screen	Compound	Name
1		SC030
2		SC021

Table 1. Top hit compounds identified through each screen. Conserved chemotype (highlighted in blue) consisted of a carboxamide linked thiazole ring.

Methods

Bacteria. *Acinetobacter baumannii* colistin resistant strain MAC204 was previously generated by Robert Ernst (19). The *naxD* mutant in MAC204 was generated by replacing the *naxD* with a kanamycin resistance cassette, as previously described (17). Strains MU134, MU185, and MU151 were provided by the Georgia Emerging Infections Program, as part of the population based active surveillance for multi-drug resistant *Acinetobacter* infections in the Atlanta area. Strains ARCL and CI-4 were obtained from the Centers for Disease Control (27). Strains 4119 and 3942 were provided by Paige Waterman of Walter Reed Army Institute of Research (28). Strain AB15/132 was collected in the United Kingdom and provided by Neil Woolford (16). Strain C14 was collected was provided by Robert Hancock at the University of British Columbia.

Colistin susceptibility assays. Susceptibility testing was performed by broth microdilution using Mueller Hinton broth (MHB) in 96-well flat bottom microtiter plates according to CLSI guidelines. In brief, colistin sodium sulfate (Sigma) was dissolved in water and potency adjusted to achieve the desired concentrations. Inhibitor SC030 (Emory Chemical Biology Discovery Center ID# 26620219) was dissolved in DMSO and diluted to appropriated concentrations in sterile distilled water. Overnight cultures were grown in MHB at 37°C with aeration, then back diluted in MHB to obtain appropriate cell density for a final assay concentration of $5x10^5$ CFU/mL. Growth was assessed after 20 hr incubation at 37°C, without aeration.

***In vitro* screen.** High throughput screening was performed at the Emory Chemical Biology Discovery Center in 384-well plates, using 25% TSB supplemented with 0.5mM $FeCl_3$. Compounds were dissolved in DMSO and assayed at 40uM concentration. Overnight cultures of bacteria were inoculated as above for colistin susceptibility assays. Plates were incubated at 30°C

for 20hrs without aeration. Data were analyzed using Cambridge Bioassay. Where appropriate, colistin was added to the assay at a final concentration of 1 μg/mL. Primary positive hits demonstrated greater than 50% growth inhibition in the presence of colistin and final hits demonstrated greater than 3-fold inhibition when applied as a colistin-inhibitor treatment relative to an inhibitor only control.

***In silico* screen.** A homology model of *A. baumannii* NaxD (NCBI gene ID A1S_2623) was generated with Chimera (https://www.cgl.ucsf.edu/chimera/) using the crystal structure of *T. thermopilus* Ydjc family protein TTHBO29 (20). Compound structures for virtual screening were obtained from the MayBridge (https://www.maybridge.com) and Specs (http://www.specs.net/snpage.php?snpageid=home) screening compound chemical databases, and interaction of structures with the NaxD model was screened using Glide-docking requesting 5 poses per structure. Top scoring structures were saved, and compounds ordered from the respective libraries for downstream *in vitro* screening as above.

Lipid A Analysis. Lipid A was harvested and analyzed as previously described (17). In brief, bacterial cultures were grown at 37°C in Mueller Hinton broth containing 40μM SC030 (where applicable) and a 0.1X MIC subinhibitory concentration of colistin to induce maximal lipid A modification. Cultures were harvested at OD600=1.0, washed in PBS, and pellets frozen for downstream lipid A extraction. Pellets were processed by incubation at 37°C in isobutyric acid and ammonium hydroxide, pelleted, and supernatants collected for lipid A isolation by resuspension in 3:1.5:0.25 C:M:H_2O with Dowex beads (Sigma). Spectra were acquired by MALDI-TOF in negative ion mode on a Bruker microFlex and processed using flexAnalysis software.

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CHAPTER 4: Discussion and Conclusion

The rise in antibiotic resistance over the last several years has raised global healthcare concerns regarding the continued successful treatment of patients with bacterial infections. Antibiotic resistance has specifically been highlighted by public health organizations such as the Centers for Disease Control and the World Health Organization as a significant problem, warranting increased disease surveillance and a renewed research focus to help reduce long term consequences conferred by untreatable infections (1, 2). Antibiotic resistance was noted in particular as a growing problem for Gram negative bacteria that historically caused nosocomial infections and have now developed resistance to the preferred cell-wall acting antibiotics of the cephalosporin and carbapenem classes (3, 4). These types of infections, including those caused by the species *Enterobacter cloacae* and *Acinetobacter baumannii*, are associated with increased morbidity and mortality and may require treatment with the cationic antimicrobial colistin as an option of last resort (3, 5, 6). Colistin was initially introduced to the clinic several decades ago, however its usage fell out of favor after the development of alternative antibiotics with more favorable toxicity profiles (7). Unfortunately, the recent increases in nonsusceptibility to more favored classes of antibiotics, such as the carbapenems, have led to reintroduction of colistin usage and a corresponding increase in resistance to this last-line drug (8, 9).

As a whole, the insights revealed through this dissertation serve to enhance our knowledge pertaining to the biology of antibiotic resistance. In particular, this work focused the problem of colistin resistance as an opportunity to explore two complementary sides of the resistance problem by: (1) deciphering the bacterial genetics of a colistin resistance mechanism, and (2) utilizing genetic information to identify novel approaches to manipulate the expression of colistin resistance. Given that colistin has become an increasingly important antibiotic in recent years, it

is imperative that we fully understand colistin resistance mechanisms and identify methods to combat resistance in order to prolong the efficacy of this last resort drug.

One elusive form of colistin resistance that was previously noted in the literature but poorly characterized was heteroresistance. These reports of heteroresistance typically described a culture of bacteria that appeared predominantly susceptible to the antibiotic, however the appearance of breakthrough colonies growing on elevated concentrations of drug was usually noted (10). In Chapter 2, we further characterized the properties of antibiotic heteroresistance utilizing a model of colistin heteroresistant *E. cloacae.* With this model, we confirmed that only a subpopulation of cells within the bacterial culture were capable of survival and growth on high concentrations of colistin, and moreover identified that the resistant subpopulation prevalence could change in response to environmental pressures. In depth interrogation of genomic and transcriptomic data revealed that the colistin susceptible and resistant cell populations appeared genetically identical but exhibited markedly different gene expression profiles. We subsequently identified a transcriptional signature that was upregulated in the resistant population and corresponded to the PhoPQ two component regulatory system. This hallmark paved the way for identification of the sensor kinase gene *phoQ* of this two component system as being required for colistin heteroresistance. *phoQ* was also required for high-level expression of *arnB,* a gene involved in the production of aminoarabinose modifications to the outer membrane molecule lipid A, which is widely recognized as the colistin drug target. Finally, we demonstrated that *phoQ* was required for colistin treatment failure in a murine model of infection with colistin heteroresistant *E. cloacae*.

Future directions for this study will aim to characterize the population dynamics of the resistant subpopulation. Remaining questions include identifying how resistant cells arise within a population and also determining if resistance is heritable or transiently expressed in these cells.

An important point to note is that the gene *phoQ* is widely conserved throughout many species of Gram negative *Enterobacteriaceae,* including other strains of *E. cloacae* and additional ESKAPE pathogen species that include *Escherichia coli* and *Klebsiella pneumoniae* (11, 12). Given the prevalence of this gene and the apparent lack of colistin heteroresistance in all strains within these species, it is critical that we identify additional genetic factors that regulate the heteroresistance phenomenon so that a more complete understanding of colistin heteroresistance can be developed.

In addition to our work, recent publications have suggested additional mechanisms that contribute to heteroresistance. Mutations in genes that regulate the activity of PhoPQ were identified in colistin heteroresistant strains of *K. pneumoniae* (13). Gene duplication events that transiently increased the chromosomal copy number of *pmrD*, a gene that links PhoPQ to additional regulatory networks, was recently implicated in low-level colistin heteroresistance in the Gram negative species *Salmonella enterica* (14). Finally, upregulation of efflux pumps was also identified in colistin heteroresistant strains of *Enterobacter cloacae*, implying that colistin toxicity in the bacterial cell can be alleviated through export from the cellular compartment (15). Altogether, the variety of mechanisms implicated in colistin heteroresistance clearly highlight that we have not yet uncovered the complete genetic pathway for this resistance phenomenon, and paves the way for future research.

Once we understand how resistance arises, new drug targets for the development of resistance inhibitors can be identified. Compounds that inhibit bacterial resistance mechanisms serve as an alternative strategy to reduce the impact of antibiotic resistance by restoring efficacy to drugs that are currently on the market. This strategy of resistance inhibition was successfully employed with the development of clavulanic acid to inhibit beta-lactamase resistance enzymes (). To achieve full efficacy, clavulanic acid is now administered as a dual treatment in conjunction

with certain beta-lactam antibiotics to treat organisms that harbor enzymatic resistance to these drugs (16).

In Chapter 3, we utilized this historical example of resistance modulation as an example to guide our strategy in developing a small molecule inhibitor that could alter colistin resistance, thereby restoring efficacy of this critical antibiotic. Previous work from our group identified the *naxD* colistin resistance gene in ESKAPE pathogen species of *Acinetobacter baumannii,* and subsequently characterized NaxD as an enzyme that primes the addition of galactosamine residues onto the lipid A molecule in the outer membrane, resulting in elevated resistance to colistin (17). Given that *naxD* mutants of *A. baumannii* display increased susceptibility to colistin, we sought to develop inhibitors of NaxD that could reverse colistin resistance and restore colistin efficacy against resistant strains of *A. baumannii.* To do so, we employed a combinatorial strategy to identify *naxD* inhibitors that would act as antibiotic adjuvants to restore colistin susceptibility in a pre-existing colistin resistant strain. Tandem high throughput screens were utilized to enrich for compounds that exhibited both (1) synergistic activity with colistin and (2) high predicted interaction with NaxD. Through these screens, a conserved inhibitor chemotype was identified, and one molecule that was representative of this chemotype successfully repressed colistin resistance with a high degree of potency, but exerted no effect on a *naxD* mutant strain. Furthermore, we demonstrated that the inhibitor prevented galactosamine addition to lipid A, confirming that the compound indeed blocked NaxD-dependent cellular modifications required for colistin resistance. Finally, we verified that the inhibitor could successfully restore colistin susceptibility in multiple strains of *A. baumannii* isolated from human infections, emphasizing the potential for this strategy to combat colistin resistance in the clinic.

Future directions for this study will include testing of the inhibitor against additional species of bacteria where NaxD homologs have been identified, such as the intrinsically colistin resistant *Francisella* species (18). Given that the inhibitor blocks resistance modifications to lipid A, there is potential for this molecule to reduce resistance to other cationic antimicrobials that target the outer membrane, such as the cathelicidin group of antimicrobial peptides produced as an innate immune defense mechanism in many organisms. Cross-resistance between colistin and the human cathelicidin LL-37 was previously demonstrated in *A. baumannii,* therefore the potential for this new inhibitor to work in such a manner is promising (19). Despite our early successes with the NaxD inhibitor *in vitro*, much works remains to fully validate the efficacy of this compound. To build on this current study, the structure activity relationship of the inhibitor with NaxD will be further explored through targeted protein mutagenesis combined with chemical modification of the inhibitor to identify which compound-protein interactions are critical for activity. These results will ultimately pave the way for future development of a modified inhibitor that exhibits more powerful activity.

In conclusion, this body of work ultimately defines a new paradigm in the antibiotic resistance field through characterization of a previously vague resistance mechanism, while simultaneously increasing our knowledge of colistin resistance and highlighting a pathway forward to help combat this global healthcare crisis.

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APPENDIX I: Antimicrobial Peptide Resistance Mechanisms of Gram-Positive Bacteria

Antimicrobial Peptide Resistance Mechanisms of Gram-Positive Bacteria

by

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Abstract

Antimicrobial peptides, or AMPs, play a significant role in many environments as a tool to remove competing organisms. In response, many bacteria have evolved mechanisms to resist these peptides and prevent AMP-mediated killing. The development of AMP resistance mechanisms is driven by direct competition between bacterial species, as well as host and pathogen interactions. Akin to the number of different AMPs found in nature, resistance mechanisms that have evolved are just as varied and may confer broad-range resistance or specific resistance to AMPs. Specific mechanisms of AMP resistance prevent AMP-mediated killing against a single type of AMP, while broad resistance mechanisms often lead to a global change in the bacterial cell surface and protect the bacterium from a large group of AMPs that have similar characteristics. AMP resistance mechanisms can be found in many species of bacteria and can provide a competitive edge against other bacterial species or a host immune response. Gram-positive bacteria are one of the largest AMP producing groups, but characterization of Gram-positive AMP resistance mechanisms lags behind that of Gram-negative species. In this review we present a summary of the AMP resistance mechanisms that have been identified and characterized in Gram-positive bacteria. Understanding the mechanisms of AMP resistance in Gram-positive species can provide guidelines in developing and applying AMPs as therapeutics, and offer insight into the role of resistance in bacterial pathogenesis.

1. Introduction

Antimicrobial peptides (AMPs) and the bacterial resistance mechanisms against them have been co-evolving for eons. A diverse array of life forms can produce AMPs, which can be used to promote immune defenses, nutrient acquisition or elimination of rival organisms from the environment. As a result, AMPs are found in a multitude of environments, ranging from mammalian tissues to soil and aquatic environments. This ubiquitous presence of AMPs in the environment provides strong selective pressure to drive the development of bacterial resistance against these peptides.

AMPs are typically small, charged, amphipathic molecules that can be produced in a variety of structures. Though structurally diverse, most AMPs work by interacting with the bacterial cell surface, followed by disruption of cellular integrity. Accordingly, the majority of bacterial resistance mechanisms function by limiting the interaction of AMPs with the bacterial cell surface. Mechanisms of AMP resistance include trapping or sequestering of peptides, outright destruction of AMPs by proteolysis, removal of AMPs from the cell via active transport, and structural modification of the cell surface to avoid interaction with AMPs. Many of these resistance mechanisms are upregulated in response to AMPs, allowing the bacteria to adaptively counter the effects of AMPs. Loss of these resistance mechanisms can impair the ability of bacteria to colonize plant or animal hosts and can attenuate virulence for many pathogens. Mechanisms of resistance may evolve specifically within a bacterial lineage or be genetically transferred from other AMP-resistant organisms.

In this review, we evaluate the available literature on Gram-positive bacterial resistance mechanisms to antimicrobial peptides. This review highlights methods of AMP resistance based on mode of action and location within the Gram-positive bacterial cell. We begin with an

overview of resistance mechanisms that act on AMPs extracellularly, and then discuss bacterial cell surface alterations. Finally, we consider removal of AMPs from the bacterial cell via transport.

2. Extracellular Mechanisms of Resistance: Enzymatic Degradation and AMP Blocking

The initial site of AMP interaction is at the bacterial cell surface. As a result, extracellular mechanisms of AMP inactivation have evolved as a first line of defense to minimize damage to the bacterial cell. Extracellular AMP resistance mechanisms have arisen in two main forms: enzymatic inactivation and sequestration (see Table 1 and Figure 1). The majority of these direct targeting mechanisms have evolved to recognize cationic AMPs. Cationic AMPs are positively charged peptides that may differentially target negatively charged moieties on the outer cell envelope, including teichoic acids, lipid II, and phosphatidylglycerol [1–3].

2.1. Extracellular Proteases

The degradation of AMPs by proteases is a mechanism of resistance found in many Gram-positive species, including *Enterococcus faecalis*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* [4–6]. AMP-degrading proteases generally have broad substrate specificity, are typically found in mammalian pathogens, and include both metallopeptidases and cysteine proteases [7,8]. This section will present several examples of AMP-degrading proteases produced by Gram-positive bacteria and detail their effects on resistance.

AMP-degrading proteases are often secreted by bacteria into their surrounding extracellular environments. Gelatinase, an extracellular metallopeptidase produced by some strains of the opportunistic pathogen *E. faecalis*, cleaves the human cathelicidin, LL-37, resulting in the loss of

antimicrobial activity *in vitro* [4]. The production of gelatinase by *E. faecalis* is associated with bacterial dissemination in animal models of disease and with increased incidence of dental caries in humans [9,10]. One example of a secreted protease made by *S. aureus* that confers AMP resistance is the aureolysin enzyme [5]. Aureolysin can hydrolyze the C-terminal bactericidal domain of LL-37, rendering the AMP inactive [11]. An infection model using human macrophages revealed that aureolysin contributes to Staphylococcal persistence within the phagosomal compartment [12], an environment that contains high levels of the antimicrobial peptide, LL-37 [13]. Additionally, some species of Staphylococci possess proteases that combat anionic AMPs such as dermcidin, a negatively charged peptide secreted by human sweat glands [14]. SepA (or SepP1) made by *S. epidermidis*, is a secreted metalloprotease that can cleave and inactivate dermcidin [6,15]. The SepA protease appears to specifically target dermcidin *in vitro* [6,16].

Gram-positive proteases are also capable of targeting AMPs at the bacterial surface. SpeB is a cysteine proteinase secreted by the pathogenic bacterium *Streptococcus pyogenes* [17]. SpeB has broad substrate specificity and cleaves AMPs, such as LL-37, and other host proteins such as fibrin, immunoglobulins, and other immune modulators [4,18–21]. In an example of adaptive resistance, SpeB was found to complex with the host α_2-macroglobulin (α_2M) proteinase inhibitor during infection [22]. The catalytically active SpeB-α_2M complexes are retained on the bacterial cell surface by association with the *S. pyogenes* G-related α_2M-binding protein (GRAB) [22,23]. The SpeB-α_2M complex has higher proteinase activity against LL-37, relative to free SpeB, and reduces killing of *S. pyogenes in vitro* [22].

2.2. Protein-Mediated Sequestration

Sequestration is another extracellular mechanism of AMP resistance [24–29,101]. Some Gram-positive bacteria produce extracellular or surface-linked proteins that directly bind to AMPs and block access to the cell membrane. Mechanisms of protein-mediated AMP sequestration vary between species and strains. We have highlighted specific examples of AMP sequestration mechanisms identified amongst strains of *S. pyogenes*, *S. aureus*, *Streptococcus agalactiae*, and *Lactococcus lactis*.

Proteins that inhibit AMP activity through binding can be secreted into the extracellular environment to inhibit contact of bactericidal peptides with the cellular surface. For example, the Streptococcal inhibitor of complement (SIC) produced by *S. pyogenes* is a hydrophilic, secreted protein that sequesters many AMPs, thereby preventing them from reaching cell-surface targets [102]. SIC binds to α-defensins, LL-37, and lysozyme, neutralizing the AMPs and inhibiting their bactericidal activity against *S. pyogenes* [27,102,103]. SIC production promotes bacterial survival *in vitro* and increases the virulence of *S. pyogenes* in animal models of disease [26,104]. Staphylokinase secretion by *S. aureus* is another example of an extracellular AMP resistance mechanism. Production of the staphylokinase protein by *S. aureus* occurs through the lysogenic conversion of the hlb β-hemolysin toxin gene by a bacteriophage harboring the *sak* gene [105–107]. Staphylokinase binds the murine cathelicidin mCRAMP *in vivo* and also complexes with human defensins HNP-1 and HNP-2 to reduce their bactericidal effects [28,29]. Studies of staphylokinase binding suggest that the staphylokinase-cathelicidin complex promotes host tissue invasion by activating the conversion of plasminogen to the host extracellular matrix-degrading enzyme, plasmin, although the role this conversion plays in Staphylococcal virulence remains unclear [29,101,108].

Proteins attached to the cellular surface can also bind AMPs to prevent contact with cell-associated targets. Examples of such proteins include the M1 protein of *S. pyogenes* and the pilus subunit, PilB of *S. agalactiae*. M1 of *S. pyogenes* can be found on the surface of most clinical isolates and has been linked to both host tissue adherence and invasive disease [109]. A hyper-variable extracellular portion of the M1 protein was shown to bind LL-37 and prevent the AMP from reaching the cell membrane [24]. The sequestration of LL-37 by M1 also promotes Streptococcal survival in neutrophil extracellular traps (NETs) by reducing LL-37 activity [24]. Like the M proteins of *S. pyogenes*, pili are also associated with invasive disease and promotion of host cell adherence by *S. agalactiae* [110,111]. Pili are large, filamentous, multimeric protein complexes expressed on the cell surface of *S. agalactiae* and other bacteria. Expression of the Streptococcal pilin subunit, PilB, promotes association of LL-37 with the bacterial cell surface and correlates with increased resistance to the murine cathelicidin mCRAMP *in vitro* [25]. In addition, pilB mutants of *S. agalactiae* (GBS) exhibit reduced fitness relative to wild-type strains in murine infection models [25]. These data suggest that in addition to the adhesin properties of pili, pilus-mediated binding of AMPs also contributes to *S. agalactiae* virulence within the host.

Another family of membrane-associated AMP resistance proteins encompasses the LanI immunity proteins of some bacteriocin producer strains. LanI proteins are typically encoded near a bacteriocin biosynthetic operon and provide protection against the bacteriocin made by the producer bacterium [112,113]. LanI-type immunity proteins are lipoproteins that anchor to the bacterial cell surface and confer resistance by either binding directly to AMPs or outcompeting AMPs by binding directly to the cellular target [114–117]. The LanI lipoproteins often work in concert with LanFEG transporters, possibly acting as substrate-binding partners for specific lantibiotics. The best characterized of the transporter-associated LanI proteins are the NisI and

SpaI lipoproteins found in strains of *L. lactis* and *Bacillus subtilis*, respectively [36,37,118] (described in transporter section). But, several lantibiotic producers encode only a LanI immunity protein and do not encode an apparent LanFEG transporter (e.g., PepI of *S. epidermidis* [119], lactocin S [120] of *L. sakei* and epicidin 280 of *S. epidermidis* [121]). In these systems, the LanI lipoprotein confers full immunity to the associated lantibiotic. Though some LanI structures have been characterized, LanI lipoproteins generally have low, if any, homology to one another [116,122]. Thus, it is unclear if mechanism of action for LanI-mediated immunity is conserved between different LanI lipoproteins.

2.3. Inhibition of AMP Activity by Surface-Associated Polysaccharides

Extracellular polysaccharide production has long been recognized as a factor that promotes both virulence and host colonization by many bacteria [123–125]. Extracellular polysaccharides are composed of structurally diverse polymers that are enzymatically produced by some Gram-positive species [126,127]. Extracellular polysaccharides that are attached to the cellular surface through covalent linkages with the cell wall are known as capsules (capsular polysaccharide, or CPS), while loosely attached polymers are referred to as exopolysaccharides, or EPS [128–130]. Polysaccharide-mediated AMP resistance is thought to occur by shielding the bacterial membrane via binding or electrostatic repulsion of AMPs [34,131].

The production of capsular polysaccharides provides resistance to a variety of AMPs and other antimicrobials and can allow some bacteria to evade host detection. Capsule-AMP binding can be mediated by the electrostatic interaction of positively charged AMPs with the negatively charged polysaccharide capsule [32]. For example, free capsular extracts from *Streptococcus pneumoniae* bind both polymyxin B and the α-defensin HNP-1, preventing these AMPs from

reaching the cell membrane and promoting bacterial survival *in vitro*. Additionally, both polymyxin B and HNP-1 promote release of the capsule from *S. pneumoniae* without loss of cell viability, suggesting that capsule release may be a mechanism of AMP resistance by sequestering AMPs away from the bacterial cell surface [32]. In another example, production of the exopolysaccharide intercellular adhesion, PIA, by *S. epidermidis* reduces killing by human defensin hBD-3, cathelicidin (LL-37), and the anionic AMP dermcidin. PIA is hypothesized to shield the bacterial membrane from the effects of AMPs [33,34]. Predictably, PIA production is associated with *S. epidermidis* virulence in multiple animal infection models [132,133]. However, while many exopolysaccharide capsules can provide resistance to AMPs, this protection is not universal to all capsule-producing Gram-positive bacteria [134–136].

3. Membrane and Cell Wall Modifications

The bacterial cell wall and membrane comprise a major target for the bactericidal activity of AMPs [137–139]. Bacteria frequently modify cell surface components to counter the effects of AMPs by reducing the net negative charge of the cell, altering membrane fluidity, or directly modifying AMP targets [140–142].

3.1. Repulsion of AMPs

Many AMPs target bacterial cells through electrostatic interactions with the cell surface [137–139]. The net charge of the bacterial cell surface is generated by anionic components of the cell membrane and cell wall, such as phospholipids and teichoic acids [143–145]. In turn, positively charged AMPs are attracted to the negatively charged bacterial cell surface [144,145]. Hence, a

broad strategy of resistance to positively charged AMPs is to alter the components on the cell surface to decrease the net negative charge of the cell, thereby limiting the electrostatic interaction of AMPs with the bacterial cell surface.

One component of the bacterial cell membrane that carries a negative charge is phosphatidylglycerol [144,145]. But in many Gram-positive bacteria, the negative charge on phosphatidylglycerol can be masked via the addition of a positively charged amino acid by the multipeptide resistance factor protein, MprF [146,147]. MprF is an intergral lysyl-phosphatidylglycerol synthetase that synthesizes and translocates aminoacylated-phosphatidylglycerol to the external membrane layer of the bacterial cell. MprF synthases were initially found to incorporate a positively charged lysine into phosphatidylglycerol (Lys-PG), decreasing the net negative charge on the bacterial membrane. In *S. aureus*, *Listeria monocytogenes*, *E. faecalis*, *Enterococcus faecium*, *B. subtilis*, and *Bacillus anthracis*, the aminoacylation of phosphatidylglycerol by MprF confers resistance to positively charged AMPs [47–49,148–150]. Additionally, an MprF homolog is present in *Mycobacterium tuberculosis*, which also confers resistance to positively charged AMPs. This MprF homolog, LysX, carries out the same functions as MprF, with the addition of a lysyl-tRNA synthetase activity [46,151]. Lysinylation of phosphatidylglycerol confers resistance to a broad spectrum of AMPs, including human defensins, gallidermin, nisin, lysozyme, daptomycin, polymyxin B, and vancomycin (Table 1) [46,150–159]. In addition to lysine modifications, some MprF orthologs can modify membrane phosphatidylglycerol with multiple amino acids, including alanine and arginine [149,160]. The enhanced antimicrobial resistance provided by aminoacylation of phosphatidylglycerol is also associated with increased virulence for several Gram-positive pathogens [46,48,49,161,162].

The Dlt pathway is another enzymatic mediator of AMP resistance that has been identified and studied in many Gram-positive genera including *Staphylococcus*, *Listeria*, *Enterococcus*, *Bacillus*, *Clostridium*, *Streptococcus*, and *Lactobacillus* [2,40–45,163–168]. The enzymatic functions of the DltABCD proteins lead to the D-alanylation of teichoic acids and lipoteichoic acids of the cell wall [169]. The addition of D-alanine to the backbone of teichoic acids can mask the negative charge present along these glycopolymers, thereby leading to increased surface charge and lower attraction of positively charged antimicrobials [169]. Similar to MprF, D-alanylation of teichoic acids by the Dlt pathway leads to a global change in charge distribution across the cell surface, allowing resistance to a broad range of cationic AMPs including vancomycin, nisin, gallidermin daptomycin, polymyxin B, lysozyme, and cathelicidins [2,39,141,163,166,170–172].

In addition to charge modification of teichoic acids, high-resolution microscopy of Group B *Streptococcus* revealed that D-alanylation could increase cell wall density, leading to increased surface rigidity [173]. Accordingly, D-alanylation may confer resistance to AMPs both by reducing the electrostatic interactions between AMPs and the cell surface and by decreasing the permeability of the cell wall [173]. As AMPs are ubiquitous within animals, D-alanylation of the cell wall can affect host colonization for pathogens and non-pathogenic species [41,152,164,174,175].

3.2. Target Modification

The cell wall is a common antimicrobial target for Gram-positive organisms. As a result, bacteria have evolved multiple modifications that limit antimicrobial targeting of the cell wall. Lysozyme, or *N*-acetylmuramide glycanhydrolase, an antimicrobial enzyme, is an important component of the host innate immune defense. Lysozyme is cationic at physiological pH, which

facilitates its interaction with negatively charged bacterial surfaces. The cationic and muramidase activities of lysozyme directly target the bacterial peptidoglycan, the primary constituent of the cell wall [176]. The muramidase domain of lysozyme hydrolyzes the β-1,4 linkages between *N*-acetylglucosamine and *N*-acetylmuramic acid of peptidoglycan, leading to the breakdown of the peptidoglycan macromolecular structure and eventual lysis of the cell [177–179]. As a result, bacterial resistance mechanisms have evolved to counter both the muramidase and cationic activities of lysozyme. In this section, we detail the mechanisms by which peptidoglycan is modified to limit lysozyme activity.

Two peptidoglycan modifiers that contribute to AMP resistance in some Gram-positive bacteria are the enzymes PgdA and OatA. It is proposed that the modifications made by both of these enzymes lead to steric hindrance between AMPs and the cell surface, thereby limiting the contact between lysozyme and its target [180]. PgdA deacetylates *N*-acetylglucosamine residues of peptidoglycan, generating a less favorable substrate for lysozyme [181–184]. PgdA was first implicated as a peptidoglycan deacetylase in the respiratory pathogen *S. pneumoniae*. PdgA and other peptidoglycan deacetylase orthologs have been shown to contribute to AMP resistance in many bacteria, including *Enterococcus*, *Streptococcus*, *Listeria* and *Bacillus* species [56–58,180,183 55,185]. Moreover, deacetylation of peptidoglycan enhances colonization and virulence in several pathogens, including *E. faecalis*, *L. monocytogenes* and *S. pneumoniae* [185–187]. As *N*-acetylglucosamine deacetylases are encoded within the genomes of most Gram-positive bacteria, these enzymes likely contribute to lysozyme and host colonization in many more species.

OatA (also known as Adr in *S. pneumoniae*) is another type of peptidoglycan modifying enzyme found in Gram-positive bacteria that confers resistance to lysozyme [188–190]. OatA

performs *O*-acetylation at the C6-OH group of *N*-acetylmuramyl residues in peptidoglycan [188–190]. *O*-acetylation of *N*-acetylmuramyl residues is thought to prevent lysozyme from interacting with the β-1,4 linkages of peptidoglycan by steric hindrance [180]. OatA and orthologous proteins have been characterized in *Staphylococcus*, *Enterococcus*, *Lactococcus*, *Bacillus*, *Streptococcus* and *Listeria* species [51,52,54,58,180,187,191]. Like deacetylation mechanisms, *O*-acetylation of peptidoglycan is likely to be widespread among Firmicutes and has been noted to contribute to virulence in animal models of infection [52,54,187,190,192].

A peptidoglycan modifier unique to *Mycobacterium* is the enzyme NamH (*N*-acetylmuramic acid hydroxylase). NamH hydroxylates *N*-acetylmuramic acid residues leading to the production *N*-glycolylmuramic acid. The modification of peptidoglycan by NamH was determined to confer lysozyme resistance in *Mycobacterium smegmatis* [59]. It is likely that NamH confers lysozyme resistance to Mycobacterial species through the generation of *N*-glycolylmuramic acid, as NamH is well conserved in Mycobacterial genomes. It is hypothesized that *N*-glycolylmuramic acid residues may stabilize the cell wall; however, the mechanism of resistance is not fully understood [193]. However, recent work suggests that the presence of an *N*-glycolyl group blocks lysozyme from accessing the
β-1,4 peptidoglycan bonds, preventing the muramidase activity of lysozyme and leaving the cell wall intact [59].

3.3. Alterations to Membrane Order

Apart from AMP repulsion and AMP target modifications as mechanisms of resistance, other changes in membrane composition can also reduce the susceptibility of bacteria to AMP-mediated killing. Alterations in Gram-positive membrane composition appear to contribute to

AMP resistance by affecting the peptide interactions with the cell membrane. In particular, the degree of membrane fluidity appears to be an important determinant of AMP susceptibility.

One example of a membrane alteration that confers AMP resistance is the saturation of membrane fatty acids. Investigations into the cell membrane components of nisin-resistant *L. monocytogenes* showed that some resistant strains contained a higher proportion of saturated (straight chain) fatty acids versus unsaturated (branched chain) fatty acids [194,195]. Additionally, a nisin resistant strain of *L. monocytogenes* produced lower concentrations of the lipid head group phosphatidylglycerol and less diphosphatidylglycerol than a nisin-susceptible strain [194–196]. This nisin-resistant strain also contained higher concentrations of the lipid head group, phosphatidylethanolamine, while the anionic membrane component, cardiolipin, was decreased [197]. These studies suggest that higher concentrations of saturated fatty acids, a decrease in phophatidylglycerol and an increase in phophatidylethanolamine head groups in the *Listeria* membrane lead to a decrease in cell membrane fluidity [194–197]. It is proposed that the decrease in membrane fluidity increases nisin resistance by hindering nisin insertion into the membrane [197].

The addition of other membrane components can also increase rigidity and lead to resistance to host AMPs and daptomycin in *S. aureus* [198]. Increased membrane rigidity in some Gram-positive organisms can result from carotenoid overproduction [199,200]. Carotenoids are organic pigments made of repeating isoprene units that are produced by plants, bacteria, and fungi [201]. Carotenoids, such as staphyloxanthin made by *S. aureus,* can stabilize the leaflets of the cell membrane by increasing order in the fatty acid tails of membrane lipids and lead to decreased susceptibility to AMPs [199,202,203]. This stabilization of fatty acid tails leads to an increase in

cell membrane rigidity, which is suggested to limit insertion of AMPs into the membrane [204,205].

Though a higher concentration of saturated fatty acids in the membrane confers AMP resistance in some bacteria, other bacteria increase unsaturated fatty acid concentrations to increase resistance. In *S. aureus*, increased levels of unsaturated membrane lipids increase the resistance to the host AMP, tPMP (thrombin-induced platelet microbicidal proteins) [206]. Unsaturated fatty acids contain double bonds along the length of their carbon chain, which causes lipid disorder, thereby increasing membrane fluidity and impacting resistance to antimicrobials [206,207]. Other studies in AMP resistance found that methicillin-resistant *S. aureus* isolates that developed resistance to daptomycin also had increased resistance to host tPMPs and the human neutrophil peptide, hNP-1. These co-resistant strains have a phenotype defined by increased cell wall thickness and increased membrane fluidity [198].
It is hypothesized that these altered membrane arrangements may prevent efficient AMP insertion into the membrane [198,206,207].

At present, there is no clear explanation as to how alterations in membrane fluidity or rigidity lead to AMPs resistance. From the examples discussed above, it could be argued that the degree of fluidity required for resistance to a particular AMP may be as varied as the structures of the AMPs themselves, or perhaps is constrained to groups with similar mechanisms of action.

4. AMP Efflux Mechanisms

Transport, or efflux, is a common mechanism used by Gram-positive bacteria for the removal of toxic compounds and antimicrobials from cells. The majority of antimicrobial peptide efflux

mechanisms consist of multi-protein ABC (ATP-binding cassette) transporter systems, which use ATP to drive the transport of substrates across or out of the cell membrane [208]. There are three primary types of ABC transporter systems implicated in Gram-positive AMP resistance: three-component ABC-transporters, two-component ABC-transporters, and single protein multi-drug resistance transporters, or MDR

pumps [209]. All ABC-transporters are composed of two distinct domains: the transmembrane domain (permease) and the nucleotide-binding domain (NBD), which facilitates ATP-binding [209]. A less common efflux mechanism that has been identified is the Major Facilitator (MFS) Transporter module, which facilitates small solute transport via a chemiosmotic ion gradient [210]. This section will present the key types of AMP transporters found in Gram-positive bacteria and highlight the AMP resistance characteristics of these systems.

4.1. Three-Component (LanFEG) Transporter Systems

Three-component ABC transporters, or LanFEG systems, are best characterized in AMP-producing bacteria. LanFEG systems are members of the ABC-type 2 sub-family of transporters, and consist of one protein with a nucleotide-binding domain (LanF) and two distinct transmembrane permeases (LanE and LanG) [211]. The majority of the characterized LanFEG systems are self-immunity mechanisms that provide protection against bacteriocins (typically lantibiotics) made by bacteriocin producer strains [38,112] (Table 1). The LanFEG transporters are often found in conjunction with LanI membrane-associated lipoproteins that can function in tandem with the transporter to provide greater resistance to AMPs [112,212,213].

The best-characterized LanFEG transporters are the NisFEG and SpaFEG systems found in strains of *L. lactis* and *B. subtilis* that produce the lantibiotic AMPs nisin and subtilin,

respectively. Both NisFEG and SpaFEG provide resistance to their cognate substrates, but full resistance is achieved in concert with their associated substrate-binding lipoproteins, NisI and SpaI [100,213–215]. Immunity to the lantibiotic nukacin ISK from *Streptococcus warneri* does not involve a LanI protein, but instead contains a distinct membrane-associated protein termed NukH [96,216]. In contrast to the LanI proteins, NukH is not a lipoprotein; however, NukH does appear to function as a substrate-binding partner to the NukFEG transporter. Similar to LanI, NukH confers partial immunity to nukacin ISK, but full immunity requires the complete NukFEGH system [216,217].

Most characterized LanFEG systems confer resistance only to the AMP made by a producer strain, although examples have been identified that provide resistance to multiple AMP substrates in non-producer bacteria. In *Clostridium difficile*, the CprABC transporter (a LanFEG ortholog) confers resistance to nisin, gallidermin, and likely other structurally dissimilar lantibiotic peptides [85,86]. The regulation of immunity and AMP biosynthetic genes are typically coupled in bacteriocin producer strains [112]. The ability of the CprABC system to confer resistance to multiple unrelated peptides may result from the uncoupling of the immunity mechanism from bacteriocin synthesis. But non-producers that have immunity genes in the absence of AMP biosynthetic operons can have relaxed substrate specificity that allows for recognition of multiple bacteriocins. Thus, Lan transporter cross-immunity to multiple AMPs could provide a significant competitive advantage to non-producer bacteria. Indeed, a homology search for LanFEG proteins reveals that the genomes of many other Firmicutes encode predicted bacteriocin transporters that are not coupled with apparent bacteriocin synthesis genes. Hence, like other antibacterial resistance mechanisms, the LanFEG systems have found their way into non-producing species [85,86].

4.2. Two-Component ABC-Transporter Systems

Two-component ABC-transporters make up the majority of transporter-mediated AMP resistance characterized in non-AMP producing bacteria. The canonical two-component ABC-transporter consists of one nucleotide-binding protein and a separate membrane-spanning permease [218,219]. Unlike most LanFEG systems, two-component transporters often provide resistance to multiple AMPs and are common among Gram-positive bacteria. As outlined in Table 1, numerous examples of these transporters have been identified that can provide resistance to AMPs produced by humans and bacteria, including cyclic peptides and some non-peptide antibiotics [218,220].

There are two main types of two-component ABC-transporter systems that confer resistance to AMPs among Gram-positive bacteria. The first and most common type is often referred to as the BceAB group [218,221]. BceAB transporter systems contain an archetypal ATP-binding protein of about 225–300 amino acids and a larger permease component that ranges in size from 620–670 amino acids. The prototype of this transporter group, BceAB, was first identified as a bacitracin resistance mechanism in *B. subtilis* [67,68]. Since the identification of BceAB, dozens of similar transporters have been discovered in pathogenic and non-pathogenic Gram-positive species, including *S. aureus*, *L. monocytogenes*, *S. pneumoniae*, and *L. lactis* (see Table 1 for examples) [62,71,77,80]. Members of the BceAB group have demonstrated resistance to a wide-range of bacteriocins, mammalian and fungal defensins, peptide antibiotics, and other antimicrobial compounds (Table 1). Although many of the BceAB transporters confer resistance to AMPs *in vitro*, the roles of these transporters in the virulence of pathogenic species are not known.

Another common type of a Gram-positive ABC-transporter that confers AMP resistance is the BcrAB(C) system. The BcrAB(C) transporter confers resistance to bacitracin and was originally identified in a bacitracin producer strain of *Bacillus licheniformis* [81]. BcrAB transporters can be distinguished from the BceAB systems by size and topology: BcrA is an ATP-binding cassette that ranges from about 280–320 amino acids, while the BcrB permease modules are smaller, at approximately 200–250 amino acids. BcrAB is often encoded with a third protein, BcrC (or BcrD), which allows for higher resistance to bacitracin than the BcrAB transporter alone [81,222,223]. Initially it was hypothesized that BcrC functioned as part of the BcrAB ABC-transporter, however it was later demonstrated that BcrC acts as an undecaprenyl pyrophosphate (UPP) phosphatase that competes with bacitracin for UPP [222]. The BcrAB transporters are predicted to be structurally similar to the LanFEG transporters, though the Lan systems function through two dissimilar permease components, while Bcr systems operate with only one permease subunit (BcrB) [38,82,218]. Aside from the bacitracin producer strains, BcrAB and orthologous transporters have been shown to confer resistance to bacitracin in many strains of *E. faecalis*, as well as some *Streptococcus* and *Clostridium* species [35,82,83,224].

4.3. Single Membrane Protein Antimicrobial Transporters

Multi-drug resistance (MDR) ABC-transporters are common bacterial mechanisms of resistance to peptide and non-peptide antibiotics [225]. Though these transporters are most common among characterized mechanisms for non-peptide antimicrobial resistance in Gram-positive bacteria, there are examples of MDR transporters that confer resistance to AMPs. One notable MDR AMP resistance mechanism consists of the LmrA/B proteins encoded by some *L. lactis* strains [60,226]. A LmrA MDR efflux pump was first described in a non-producer strain of

L. lactis [226]. LmrB is an ortholog of LmrA found in *L. lactis* strains that produce the bacteriocins LsbA and LsbB [60]. LmrA/LmrB are membrane proteins with six predicted transmembrane segments and a C-terminal, nucleotide-binding domain [60]. LmrA provides broad resistance against a long list of peptide antibiotics and cytotoxic compounds, while LmrB confers resistance to the two bacteriocins LsbA and LsbB [60,226]. A BLASTp homology search revealed the presence of additional orthologs of LmrA/B encoded within the genomes of hundreds of Gram-positive Firmicutes, though the function and significance of these remains unknown.

A less common type of single-protein transporter involved in antimicrobial peptide resistance is exemplified by the QacA transporter of *S. aureus* [61]. QacA is a member of the major facilitator superfamily (MFS) of membrane transport proteins, which use proton motive force, rather than ATP, to drive the efflux of substrates [227]. QacA confers resistance to a variety of toxic dyes, antiseptics and disinfectants [228,229]. In addition to cationic toxins, QacA provides resistance to thrombin-induced platelet microbicidal protein (tPMP), a host-derived antimicrobial peptide [61]. QacA-dependent tPMP resistance was found to confer a survival advantage in an animal model of infection, and increased resistance to tPMP in *S. aureus* also correlates with endocarditis in humans [61,230]. QacA orthologs have also been identified in other staphylococci, as well as in *Enterococcus* and *Bacillus* species, though the ability of these orthologs to transport AMPs is not understood [231,232].

5. Conclusions

Antimicrobial peptides are diverse in both structure and function and are produced by all forms of life. As such, AMPs are an ancient defense mechanism, and resistance mechanisms to AMPs have been selected for as long as AMPs have existed. Gram-positive bacteria are ancient producers of AMPs and as a consequence, these organisms likely developed some of the first AMP resistance mechanisms.

Herein we have detailed a wide variety of AMP resistance mechanisms found in Gram-positive bacteria (summarized in Figure 1). AMPs resistance mechanisms can be broad spectrum, such as MprF and the Dlt pathway which function by decreasing the net negative charge of the bacterial cell surface, thereby reducing the attraction for positively charged AMPs from the cell. Conversely, AMP resistance mechanisms can be highly specific and only confer resistance to a single peptide. AMP resistance mechanisms can be confined to a particular species or genus, such as NamH in *Mycobacterium*, or can be distributed among multiple species, such as the LanFEG systems. AMPs resistance mechanisms are dynamic; they can be passed from species to species via bacteriophages or horizontal gene transfer, and can change specificity and function over time through evolution [85,86,105,233]. Under selective pressure, AMP resistance mechanisms can evolve to suit the needs of a particular species in its own niche [234].

At present, many AMPs are being investigated as potential antimicrobial therapies [235–240]. AMP drug development should be carefully vetted because like any naturally-produced antimicrobial, cognate resistance mechanisms for AMPs are already present in the producer bacterium. While these resistance mechanisms may be found more frequently in producer strains, each has the propensity to be passed on to other genera or species within a shared environmental niche. Because the presence of AMPs provides high selective pressure for the acquisition of resistance, it is important to consider the potential for resistance mechanism transfer between

bacteria when developing AMPs for clinical use [241,242]. Additionally, depending on the AMP resistance mechanism that is selected for, a multitude of issues may arise if the mechanism of resistance is broad-spectrum. A broad-spectrum AMP resistance mechanism could restrict the already limited clinical treatment options for use against some Gram-positive pathogens and may undermine our own immune response by conferring resistance to our own innate immune system peptides [243].

Antimicrobial peptide resistance is not as well characterized for Gram-positive bacteria as it is for Gram-negative bacteria. Thus, it is likely that many more mechanisms of antimicrobial resistance remain to be discovered in Gram-positive species. As more AMPs are found, new Gram-positive AMP resistance mechanisms will undoubtedly be revealed.

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Conflicts of Interest

The authors declare no conflict of interest.

Table 1. Summary of Gram-positive Antimicrobial Peptides (AMP) Resistance Mechanisms.

Name	Mechanism of Action	Antimicrobial Resistance	Organisms	Reference
AMP Degradation				
Aureolysin	Protease	LL-37	*S. aureus*	[5,11]
Gelatinase	Protease	LL-37	*E. faecalis*	[4,10]
SepA	Protease	dermcidin	*S. epidermidis*	[6,16]
SpeB	Protease	LL-37	*S. pyogenes*	[4,21,22]
Sequestration/Competition for AMP target				
M Protein	Binding at surface	LL-37	*S. pyogenes*	[24]
PilB	Binding at surface	cathelicidins	*S. agalactiae*	[25]
SIC	Extracellular binding	α-defensins, LL-37, lysozyme	*S. pyogenes*	[26,27]
Staphylokinase	Extracellular binding	Cathelicidin, defensins	*S. aureus*	[28,29]
LciA	Binding at surface	Lactococcin A	*L. lactis*	[30,31]
Capsule	Binding/shielding	Polymyxin B, HNP-1	*S. pneumoniae*	[32]
Exopolysaccharide	Shielding/ Sequestration	LL-37, hBD-3, dermcidin	*S. epidermidis*	[33–35]
LanI lipoproteins	Binding or competition	lantibiotics	*L. lactis*, *B. subtilis*, other lantibiotic producers	[36–38]
Cell Surface Modifications				
DltABCD	D-alanylation of teichoic acids	daptomycin, vancomycin, nisin, defensins, protegrins	*S. aureus*, *L. monocytogenes*, *B. cereus*, *C. difficile*, *S. pyogenes*, *S. agalactiae*, *B. anthracis*, *S. suis*	[2,39–45]
MprF	Lysylation of phoshatidylglycerol	defensins, thrombin-induced platelet microbicidal protein	*S. aureus*, *L. monocytogenes*, *B. anthracis*, *M. tuberculosis*	[46–50]
OatA	Peptidoglycan O-acetylase	lysozyme	*S. aureus*, *S. epidermidis, S. lugdunensis*, *E. faecalis*, *L. monocytogenes*	[51–54]
PdgA	Peptidoglycan *N*-acetylglucosamine deacetylase A	lysozyme	*S. pneumoniae*, *E. faecalis*, *S. suis*, *L. monocytogenes*, *B. anthracis*	[55–58]
NamH	*N*-acetylmuramic acid hydroxylase	lysozyme	*M. smegmatis*	[59]

Table 1. *Cont.*

Name	Mechanism of Action	Antimicrobial Resistance	Organisms	Reference
AMP Efflux				
One-component transporter				
LmrB	ABC transporter	LsbA/LsbB	*L. lactis*	[60]
QacA	ABC transporter/ alteration of membrane structure	thrombin-induced platelet microbicidal protein (tPMP)	*S. aureus*	[61]
BceAB type				
AnrAB	ABC transporter	nisin, gallidermin, bacitracin, β-lactams	*L. monocytogenes*	[62,63]
BceAB	ABC transporter	Bacitracin [a], actagardine, mersacidin, plectasin	*B. subtilis* [a], *S. mutans*	[64–68]
BraAB	ABC transporter	nisin, nukacin ISK-1, bacitracin	*S. aureus*	[69]
PsdAB	ABC transporter	nisin, enduracidin, gallidermin, subtilin	*B. subtilis*	[66]
MbrAB	ABC transporter	bacitracin	*S. mutans*	[35]
SP0812-SP0813	ABC transporter	bacitracin, vancoresmycin	*S. pneumoniae*	[70]
SP0912-SP0913	ABC transporter	bacitracin, lincomycin, nisin	*S. pneumoniae*	[71]
VraDE	ABC transporter	bacitracin, nisin, nukacin ISK-1	*S. aureus*	[69,72–76]
VraFG	ABC transporter	nisin, colistin, bacitracin, vancomycin, indolicidin, LL-37, hBD3	*S. aureus*, *S. epidermidis*	[69,72,75,77–79]
YsaCB	ABC transporter	nisin	*L. lactis*	[80]
BcrAB type				
BcrAB(C)	ABC transporter	bacitracin	*B. licheniformis*	[81]
BcrAB(D)	ABC transporter	bacitracin	*E. faecalis*	[82,83]
LanFEG type				
As-48EFG(H)	ABC transporter	AS-48	*E. faecalis*	[84]
CprABC	ABC transporter	nisin, galidermin, other lantibiotics	*C. difficile*	[85,86]
EpiFEG(H)	ABC transporter	epidermin, gallidermin	*S. epidermidis*	[87]
LtnFE(I)	ABC transporter	lacticin 3147	*L. lactis*	[88,89]
McdFEG	ABC transporter	macedocin	*S. macedonicus*	[90]
MrsFGE	ABC transporter	mersacidin	*Bacillus sp. HIL Y-84*, *54728*	[91,92]
MutFEG	ABC transporter	mutacin II	*S. mutans*	[93]
NisFEG(I)	ABC transporter	nisin	*L. lactis*	[37,94]
NukFEG(H)	ABC transporter	nukacin	*S. warneri*	[95,96]
SboFEG	ABC transporter	salivaricin B	*S. salivarius*	[97]
ScnFEG	ABC transporter	streptococcin A-FF22	*S. pyogenes*	[98]
SmbFT	ABC transporter	Smb, haloduracin	*S. mutans*	[99]
SpaFEG	ABC transporter	subtilin	*B. subtilis*	[36,100]

[a] Confers only bacitracin resistance in *B. subtilis*.

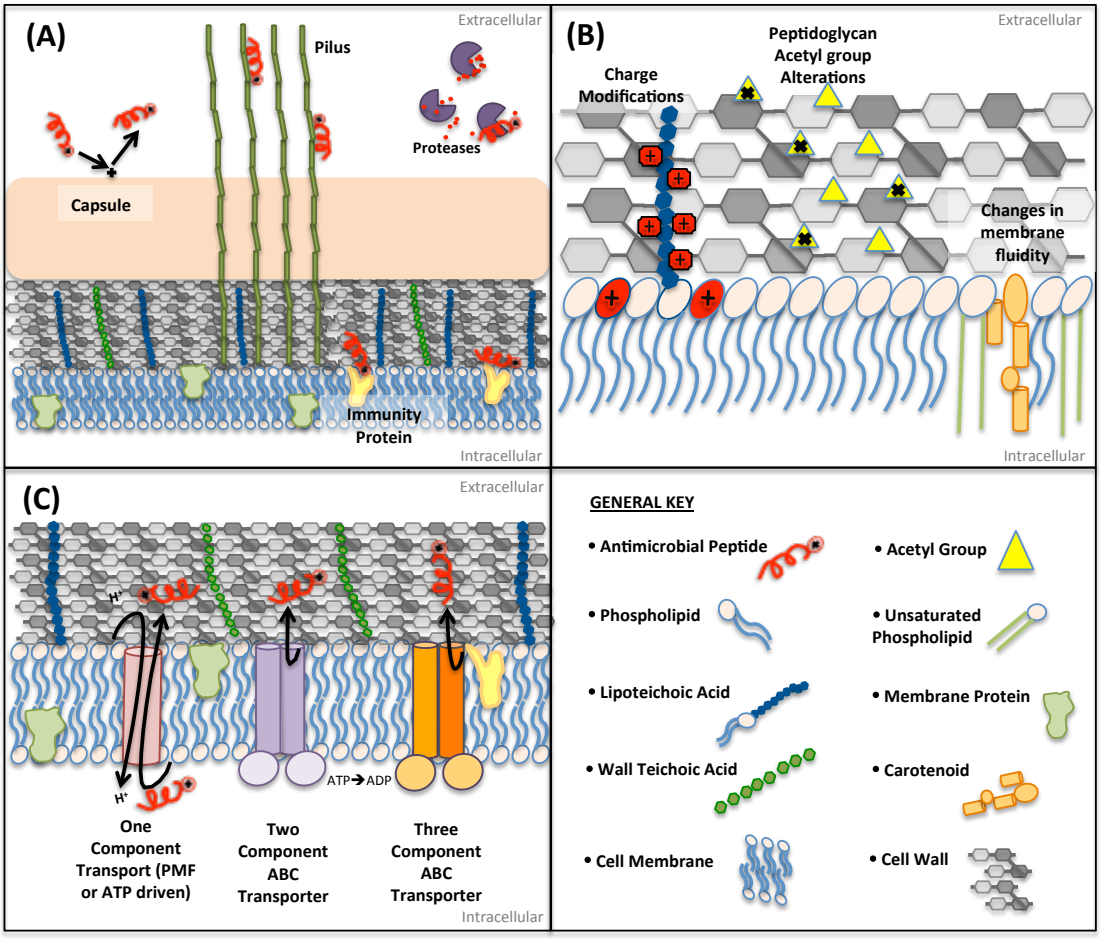

Figure 1. Overview of Antimicrobial Peptide Resistance Mechanisms in Gram-Positive Bacteria.

(**A**) Extracellular mechanisms of AMP resistance include peptide degradation by secreted proteases, AMP sequestration by secreted or membrane associated protein (e.g., pili, immunity proteins, M proteins), or blocking by capsule polysaccharides; (**B**) Cell wall and membrane modifications include: Alteration of charge by lysination of the phospholipid head groups or D-alanylation of the lipoteichoic backbone, modification of the cell wall by deacetylation of N-acetylglucosamine or O-acetylation of N-acetylmuramyl residues, and alterations in membrane fluidity by phospholipid tail saturation or carotenoid additions; (**C**) Transport mechanisms of antimicrobial efflux from the cell include: ATP-driven ABC transporters composed of a single,

double, or triple protein pump and involve a supplementary immunity protein, or single protein transporters driven by proton motive force.

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