Staphylococcus aureus COL: An Atypical Model Strain of MRSA that Exhibits Slow Growth and Antibiotic Tolerance

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Summary

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been a pathogen of global concern since its emergence in the 1960s. As one of the first MRSA strains isolated, COL has become a common model strain of *S. aureus*. Here we report that COL is, in fact, an atypical strain of MRSA that exhibits slow growth (extended lag and doubling times) and multidrug tolerance, with minimum duration of killing (MDK) values 50-300% greater than other "model" strains of *S. aureus.* Genomic analysis identified three mutated genes in COL (*rpoB, gltX* and *prs*) with links to tolerance. Allele swapping experiments between COL and the closely related, non-tolerant Newman strain uncovered a complex interplay between these genes. However, Prs (phosphoribosyl pyrophosphate [PRPP] synthetase) accounted for most of the growth and tolerance phenotype of COL. ppGpp quantitation and transcriptomic comparison of COL and Newman revealed that COL does not exhibit slow growth as a result of partial stringent response activation, as previously proposed. Instead, COL exhibits downregulation of purine, histidine and tryptophan synthesis, three pathways that rely on PRPP. Overall, our findings indicate that COL is an atypical, antibiotic-tolerant strain of MRSA whose isolation predates the previous first report of tolerance among clinical isolates.

Introduction

Staphylococcus aureus is an opportunistic pathogen and major cause of both nosocomial and community-acquired infections. It is a leading cause of many life-threatening infections, including bacteremia, pneumonia, endocarditis, and bone and joint infections[1]. Treatment of *S. aureus* infections is highly dependent on antibiotics, but *S. aureus* has an incredible capacity to develop resistance to these agents. The most widespread and clinically significant resistance occurs in methicillin-resistant *S. aureus* (MRSA). Methicillin resistance first emerged in the United Kingdom in 1960, within one year of the introduction of methicillin into clinical use (although genomic evidence suggests that MRSA actually first emerged in the 1940s[2]). Since then, MRSA has proved itself to be a formidable global pathogen, causing at least 100,000 deaths in 2019, more than any other single pathogen-drug combination[3].

Methicillin resistance in *S. aureus* is most commonly mediated by *mecA*, which is acquired horizontally as part of a mobile genetic element known as staphylococcal cassette chromosome *mec* (SCC*mec*)[4]. The *mecA* gene itself encodes for penicillin-binding protein 2a (PBP2a), which participates in peptidoglycan synthesis and has a lower binding affinity for β-lactam antibiotics than the native PBPs produced by *S. aureus*. Expression of methicillin resistance is multifactorial and supported by the presence and/or mutation of many “auxiliary” and “potentiator” genes (in addition to *mecA*)[5]. Most clinical isolates of MRSA exhibit low-level and heterogeneous resistance; the population, as a whole, exhibits a relatively low MIC, but subpopulations of cells exhibit very high levels of resistance[6]. However, some isolates – like the early MRSA isolate COL – exhibit high-level, homogeneous resistance[7]. COL (previously known as strain 9204 and MR-COL in

early literature) was one of the first MRSA strains identified, isolated in 1960 in a public health laboratory in Colindale, UK[8–10]. As a member of the so-called “archaic” lineage of MRSA[11,12], COL has become one of the most commonly used model strains of MRSA[e.g.13–21].

COL is a member of clonal complex 8 (CC8), one of the most prevalent complexes of *S. aureus* responsible for both community-acquired and healthcare-associated infections[12]. Other notable model strains belonging to CC8 include the methicillin-sensitive *S. aureus* (MSSA) strain Newman and MRSA strains of the USA300 lineage (Table 1). COL is commonly employed as a model strain of *S. aureus* [e.g.13–21], but two studies have noted that it exhibits slow growth compared with other CC8 strains[22,23]. Li et al. determined growth curves for COL and five comparator CC8 strains *in vitro* and noted that, while the growth curves of the other strains were virtually indistinguishable, COL exhibited “slightly slower growth”[22]. Later, Kim et al. also reported that COL exhibited slow growth but, more interestingly, they reported that COL maintained a higher basal concentration of the signalling molecules guanosine tetra- and pentaphosphate (collectively known as [p]ppGpp) than a comparator strain[23]. (p)ppGpp is the effector molecule of the stringent response, a universal bacterial stress response that is classically induced in response to amino acid starvation[24]. In *S. aureus*, activation of the stringent response and cellular (p)ppGpp concentration is largely controlled by a bifunctional enzyme, Rel, that both synthesises and hydrolyses (p)ppGpp[25]. Once synthesised, (p)ppGpp acts to downregulate most metabolic processes through a range of direct and indirect mechanisms in different bacteria[26]. In *S. aureus* and other members of the Firmicutes, much of the regulation of transcription and translation mediated by (p)ppGpp

is thought to occur indirectly as a result of the plummeting intracellular GTP level (Rel synthesises (p)ppGpp from GTP). The transcriptional repressor CodY requires GTP as a ligand to facilitate binding to target DNA. Therefore, a rapid decrease in the intracellular GTP pool causes CodY to be released from DNA, allowing transcription of genes associated with, for example, amino acid synthesis[27]. In contrast, the transcription of other genes is downregulated by the drop in GTP level, as many promoters (including those for rRNA genes) initiate with GTP[28]. Direct binding partners of (p)ppGpp have also been identified in *S. aureus* and closely-related bacteria, including enzymes involved in GTP synthesis and GTPases that participate in ribosome assembly[29–32]. Binding of (p)ppGpp to these protein targets inhibits their normal function, thereby contributing to the downregulation of metabolic processes.

We have previously shown that elevated cellular (p)ppGpp and a partially activated stringent response (as a result of clinical mutations in Rel) confers antibiotic tolerance in *S. aureus* Newman and USA300 strain LAC[33,34]. Antibiotic tolerance describes the ability of a bacterial population to withstand transient exposure to an otherwise lethal concentration of bactericidal antibiotic, without exhibiting an elevated minimum inhibitory concentration (MIC)[35]. As a bacterial phenomenon, genotypic tolerance was first described in the pneumococcus in 1970[36] and identified among clinical isolates of *S. aureus* in 1977[37]. It has since been detected in >20 bacterial species and, arguably most frequently, in *S. aureus*[38]. In recent years, tolerance has been strongly implicated as a contributing factor in persistent and recurrent infections[38–40] and shown to promote the development of endogenous resistance[41–43]. While stringent response activation represents one route to tolerance, numerous other molecular mechanisms have been

reported ranging from mutations in proteins associated with transcription, translation, carbon metabolism, purine biosynthesis and the electron transport chain[38]. An overarching theme that links most of these mechanisms is slow growth and reduced metabolism[33,38,44–46]. Most bactericidal antibiotics target active metabolic/cellular processes; therefore, slowing these processes down leads to a slower rate of cell death[47–51].

Given the reported slow growth phenotype of *S. aureus* COL[22,23], we hypothesised that this property may confer antibiotic tolerance (as in other strains[33,34,38,41,43]). Because COL is commonly used as a model strain of MRSA, it is important to understand any underlying mechanisms potentially impacting its behaviour in *in vitro* and *in vivo* antibiotic efficacy studies[14–16,18–21]. We, therefore, set out to investigate the tolerance profile of COL compared with other strains of *S. aureus.* Here, we show that COL exhibits a greatly extended lag phase and longer doubling time compared with other model strains of *S. aureus* and multidrug tolerance, but these phenotypes are not due to activation of the stringent response. Instead, genome analysis and allele swapping experiments demonstrate that the main contributor to the tolerance phenotype of COL is a mutation in Prs, a keystone enzyme in purine, pyrimidine, histidine, tryptophan and NAD biosynthesis. Downregulation of three of these important pathways is further evidenced by transcriptional analysis. Overall, our results reveal that COL is an atypical strain of MRSA whose isolation predates the previous first report of clinical tolerance, and that clinical antibiotic tolerance occurred in *S. aureus* around the same time as, or even before, clinical methicillin resistance.

Results

COL exhibits growth defects and multidrug tolerance

In general, two types of tolerance have been identified: tolerance by lag and tolerance by slow growth (longer doubling time in exponential phase)[33,52]. We have previously seen a combination of these two defects in a stringent response-activated mutant of *S. aureus*[33]. Li et al. reported that COL exhibited slower growth than other CC8 strains[22]. To investigate this further, we performed detailed growth curve analysis on COL and a panel of five other commonly used model strains of *S. aureus* from different CCs and sequence types (Table 1). While the mean lag times of the comparator strains didn't differ significantly from each other (Figure 1), the lag time of COL was ~50% longer than any other strain. Likewise, the mean doubling times of the comparator strains were similar, while the doubling time of COL was significantly longer.

Antibiotic tolerance has not been reported previously for COL. However, given its growth defects, we hypothesised that it would exhibit this phenotype. We performed full time-kill assays with COL and comparator strains to compare their rates of antibiotic-induced death. We used two antibiotics with different mechanisms of action (at 8 or 16 × MIC; Table 1) and for which tolerance has been observed before in other strains of *S. aureus*: daptomycin and ciprofloxacin[29,33,43]. As predicted, the rate of killing of COL by daptomycin and ciprofloxacin was significantly slower than that of the comparator strains (Figure 2A and 2B). The tolerance phenotype of COL to daptomycin was similar to that of our previously characterized tolerant *rel* mutant F128Y[33,34], while COL was more tolerant to ciprofloxacin than the *rel* mutant (Figure S1). We calculated the minimum duration for killing required to kill 99% of the population (MDK_{99}), a metric used to quantify

tolerance[35], for each model strain-antibiotic combination. While there were some small but significant differences between the comparator strains (Figure 2C and D), the MDK_{99} values for COL were 50-300% higher than all other strains. Therefore, COL appears to be an outlier with an atypical response to bactericidal antibiotics.

Genome analysis of COL vs. comparator strains

Given the unusual growth and tolerance phenotype of COL compared to other strains of *S. aureus*, we were interested to investigate the genetic basis of these differences. In line with the fact that they belong to the same clonal complex, genome analysis revealed that COL is most similar to strains Newman and LAC, with 99.7% pairwise identity to both genomes. However, this still equates to >8,000 SNPs over a 2.8 kb genome. We have previously shown that a tolerance phenotype can be accompanied by a shift towards high-level homogeneous β-lactam resistance expression[34], a phenotype that is also demonstrated by COL[7]. Therefore, in order to narrow down the pool of SNPs that may be contributing to the tolerance phenotype of COL, we considered previous work analysing the genetic basis of methicillin resistance expression in COL[7]. Kim et al. identified nonsynonymous mutations in four genes in COL that were deemed relevant to its high-level resistance expression: *prs* (ribose-phosphate pyrophosphokinase or phosphoribosyl pyrophosphate [PRPP] synthetase, involved in purine synthesis), *gltX* (glutamyl-tRNA synthetase), *rplK* (ribosomal protein L11) and *rpoB* (β-subunit of RNA polymerase)[23]. A comparison of these protein sequences between COL and the five comparator strains revealed that the D103N mutation in *rplK* in COL is also found in Newman; therefore, it is unlikely to contribute to its tolerant phenotype. The mutations in *prs* (E112K), *gltX* (E405K) and *rpoB* (A798V and S875L), however, are exclusive to COL.

Mutations in *prs*, *rpoB* and numerous tRNA-synthetases have been associated with tolerance previously[38].

Single allele swapping between COL and Newman

To experimentally test the impact of mutations in these three genes on growth and tolerance, we performed allele swapping between COL and Newman. We decided to use Newman, rather than LAC, as the comparator strain for these experiments because Newman is susceptible to ciprofloxacin which allowed us to perform time-kills with this antibiotic. We began by swapping the *prs* alleles between COL and Newman (Figure 3). Introduction of the COL *prs* allele into Newman lead to a dramatic growth defect, with a doubling time similar to that of wildtype COL and an even longer lag time. Introduction of the Newman *prs* allele into COL had no effect on doubling time, but it reduced the lag time to that of wildtype Newman. These effects were reflected in time-kills, where Newman::COL *prs* had a very similar response to ciprofloxacin as wildtype COL but was even more tolerant to killing by daptomycin than COL. With both antibiotics, the COL::Newman *prs* strain had a tolerant phenotype that was somewhere between that of wildtype Newman and COL. Interestingly, when exposed to daptomycin, COL::Newman *prs* exhibited a biphasic profile, with an initial rate of killing and MDK_{99} value indistinguishable from that of wildtype Newman (Figure 3E, Table 2) and a second, slower rate between that of Newman and COL. Collectively, these results indicate that the *prs* E112K mutation found in COL greatly contributes to its growth defects and tolerance phenotype.

Next, we moved on to the *gltX* allele and performed equivalent experiments. Introduction of the COL *gltX* allele into Newman had no effect on growth or tolerance to

ciprofloxacin (Figure 4). The response of Newman::COL *gltX* to daptomycin was also very similar to that of wildtype Newman, although killing after two hours was reduced in the mutant. When the Newman *gltX* allele was introduced into COL, we observed no effect on doubling time and only a small (but significant) impact on lag time. However, this translated into a difference in antibiotic killing. Killing of COL::Newman *gltX* by ciprofloxacin was intermediate between that of wildtype COL and Newman, while the COL::Newman *gltX* strain exhibited a kill curve with daptomycin that was very similar to that of Newman. These results suggest that while the *gltX* mutation plays a role in tolerance in the genetic background of COL, this mutation alone is not sufficient to confer tolerance in Newman.

Finally, we wanted to assess the influence of the two *rpoB* mutations on the growth and tolerance of COL. Unfortunately, attempts to introduce the COL *rpoB* allele into Newman and vice versa were unsuccessful (likely due to the large size and essentiality of *rpoB*). However, Panchel et al. have previously generated COL *rpoB*$^+$, a variant of COL in which the two *rpoB* mutations have been reversed in conjunction with the introduction of a nearby kanamycin resistance marker[53]. Therefore, we compared the growth and antibiotic killing profiles of wildtype COL and COL *rpoB*$^+$ (Figure 5). The lag times of the two strains were not significantly different, but, interestingly, the doubling time of COL *rpoB*$^+$ was slightly longer than that of wildtype COL. This small change in doubling time did not result in any difference in killing kinetics with ciprofloxacin, but COL *rpoB*$^+$ did exhibit a greater MDK_{99} for daptomycin than wildtype COL (Table 2; $P = 0.001$, unpaired two-tailed *t*-test). Therefore, the two COL *rpoB* mutations present in COL *rpoB*$^+$ do not

confer tolerance and, in fact, the genetic engineering in this strain has increased tolerance to daptomycin slightly.

Combining mutations in COL

Our single allele swapping experiments indicated that the mutation in *prs* is the major contributor to the reduced growth and tolerant phenotype of COL. However, reversing the *prs* mutation in COL did not fully abolish tolerance, and introduction of the Newman *gltX* allele into COL had a considerable effect on tolerance. Therefore, we decided to investigate the effect of multiple allele swapping in COL (Table 2, Figure S2). Replacing both the *prs* and *gltX* allelles in COL produced a strain with a lag time comparable to that of COL::Newman *prs* and a doubling time between that of COL::Newman *prs* and COL::Newman *gltX*. Its killing kinetics with both antibiotics were essentially the same as those of the *prs* single allele mutant; therefore, the effect of the *prs* allele appears to be more dominant than that of the *gltX* allele. When the *prs* allele was introduced into COL *rpoB*$^+$, the lag time was indistinguishable from that of COL::Newman *prs* and the long doubling time of COL *rpoB*$^+$ was reduced. There was no difference in the killing kinetics of COL::Newman *prs* and COL *rpoB*$^+$::Newman *prs* with daptomycin, but the effect of replacing the *prs* allele on ciprofloxacin killing was greater in the presence of the *rpoB*$^+$ allele than in wildtype COL (MDK_{99} of 1.3 h *vs* 2.5 h; Table 2). Therefore, there appears to be some combined effect of the *prs* and *rpoB* mutations. There also appears to be a complicated interaction between the *gltX* and *rpoB* alleles. Introduction of the Newman *gltX* allele into COL *rpoB*$^+$ had the same effect on lag time that it did in wildtype COL, but it abolished the extended doubling time normally exhibited by COL *rpoB*$^+$. In terms of tolerance, there appeared to be an additive effect of the *gltX* and *rpoB* mutations on

tolerance to ciprofloxacin, as COL *rpoB*$^{+}$::Newman *gltX* was killed more rapidly than COL::Newman *gltX*. However, with daptomycin, the reverse was observed and COL *rpoB*$^{+}$::Newman *gltX* was more tolerant than COL::Newman *gltX*. Finally, when both the *prs* and *gltX* alleles were introduced into COL *rpoB*$^{+}$, the resultant strain had the shortest lag time and doubling time of any COL mutant (Table 2). In terms of its tolerance profile, its killing kinetics were very similar to those of the COL *prs-gltX* double allele mutant, but it retained some tolerance to both ciprofloxacin and daptomycin compared with wildtype Newman.

(p)ppGpp quantitation and transcriptomics of COL

Our allelic exchange experiments suggest that the mutations in both Prs and GltX contribute to the tolerance phenotype of COL. Both enzymes have links with the stringent response; mutations in tRNA synthetases can limit the pool of charged tRNAs and induce (p)ppGpp synthesis[54], while the purine biosynthesis pathway (of which Prs is a keystone enzyme) produces the substrates from which (p)ppGpp is synthesised (ATP and GDP/GTP). Furthermore, COL has previously been reported to exhibit higher (p)ppGpp content than a comparator strain[23]. Therefore, we used (p)ppGpp quantitation and transcriptomics to compare the stringent response profiles of wildtype Newman and COL. Cellular extracts of exponentially-growing cultures of Newman, COL and a known stringent response-activated mutant of Newman that bears a Rel mutation, F128Y[33], were subjected to HPLC separation and analysis using an established method for quantitation of ppGpp (the most abundant and stable stringent response alarmone[55]). While F128Y had a ppGpp level significantly greater than that of wildtype Newman and COL, the ppGpp content of COL did not differ significantly from that of Newman (Figure S3).

This lack of a chemical stringent response activation signature in COL was also corroborated by transcriptomic analysis of COL *vs* Newman. The hallmarks of stringent response activation in *S. aureus* are well known and include upregulation of 150 genes under regulation by CodY[56]. We identified 147 CodY regulon genes in our COL and Newman datasets. Of these 147 genes, 105 were not differentially expressed in COL compared with Newman, 30 were upregulated in COL and 12 were downregulated in COL (Figure S4A). Therefore, there is no transcriptomic evidence to support the hypothesis that the stringent response is induced in COL or that elevated (p)ppGpp contributes to its growth and tolerance phenotypes.

A more general comparison of the transcriptomic profiles of COL and Newman revealed that many diverse categories of genes were differentially regulated (Figure 6A), including a downregulation of virulence-associated genes and upregulation of more than 20 carbohydrate-associated genes in COL. Interestingly, gene ontology enrichment analysis identified inosine monophosphate (IMP) and purine metabolism as being highly represented in the pathways upregulated in Newman compared with COL (Figure 6B). This is consistent with the pronounced effect the COL Prs mutation had on growth in our earlier experiments. Prs is a ribose-phosphate pyrophosphokinase that synthesises PRPP from ribose-5-phosphate. PRPP is a keystone metabolite as it is essential not only for *de novo* purine synthesis, but also the purine salvage pathway and the synthesis of tryptophan, histidine, pyrimidine and NAD (Figure S5)[57]. Closer analysis of the expression of genes downstream of *prs* revealed that some genes from the histidine and tryptophan pathways, and 10 of the 11 genes associated with IMP synthesis from PRPP (which are part of a single operon), were significantly downregulated in COL compared with Newman

(≥2-fold change, FDR-corrected $p<0.05$; Figure 6C and Figure S6). In contrast, genes from the pyrimidine, NAD and purine salvage pathways were not differentially expressed. Interestingly, the genes involved in the nucleotide-specific branches leading from IMP to GTP and ATP were also not differentially expressed. Therefore, downregulation of IMP, histidine and tryptophan synthesis appears to be the main transcriptomic difference between COL and Newman.

Discussion

COL is an archaic MRSA isolate that is frequently used as a model strain in *in vitro* and *in vivo* studies of *S. aureus*[14–16,18–21]. It has previously been identified as exhibiting high-level, homogeneous methicillin resistance expression, which is in contrast to most MRSA strains that exhibit low-level, heterogeneous resistance[6]. Here, we have confirmed two previous reports[22,23] that COL exhibits growth defects compared with other strains of *S. aureus* and have further shown that these defects lead to antibiotic tolerance. The slow growth of COL has previously been explained by an elevated intracellular concentration of (p)ppGpp compared with a comparator strain[23]. However, our ppGpp quantitation experiments suggest that COL contains a similar basal level of ppGpp to its genetically close comparator Newman. Further, the transcriptomic profile of COL does not show the hallmarks of stringent response activation.

While closely related, there are still many genetic differences between Newman and COL, but our allele swapping experiments focused on three genes that bear mutations in COL and that have been previously associated with tolerance: *rpoB, gltX* and *prs.* In isolation, the *rpoB* mutations A798V and S875L do not play a significant role in the tolerance phenotype of COL as their reversal had no effect on antibiotic killing.

However, there appeared to be a small additive effect of these mutations when combined with the mutant alleles of *prs* or *gltX* (although these experiments are potentially confounded by the use of the marked COL *rpoB*$^+$ strain, which grows slower than wildtype COL). Similarly, there is clearly an interplay between the GltX E405K mutation and the wider genetic background of COL in terms of tolerance. While the GltX mutation alone was insufficient to confer tolerance in Newman, reversal of this mutation in COL impacted antibiotic killing and reversal in COL *rpoB*$^+$ had an even greater impact. Testing of the different single, double and triple allele-swapped mutants of COL also revealed some differences in tolerance and killing between ciprofloxacin and daptomycin, highlighting the importance of testing more than one antibiotic. Interestingly, the COL triple mutant, with all three mutant alleles replaced, still exhibited some low-level tolerance to both antibiotics compared with Newman. Therefore, while we could conclude that these three genes may not represent the full genetic complement of tolerance in COL, tolerance is a not an absolute property but a relative phenotype and is only detected by comparison with a non-tolerant strain (in this case Newman). It should also be noted here that while the *prs* and *gltX* mutations in COL have been linked to its high-level, homogeneous methicillin resistance phenotype[23], reversal of the two *rpoB* mutations alone is sufficient to abolish this phenotype[53].

Of the three genes we looked at, the E112K Prs allele accounts for a large proportion of the extended lag time and tolerance phenotype of COL, as well as seemingly dominating its transcriptomic profile. Prs is a biochemically well-studied enzyme that is highly conserved in *S. aureus*. Bioinformatic analysis of the *S. aureus* Prs sequences in GenBank reveals that 99.1% are identical; of the 59 unique sequences, all but one has a

glutamate at position 112 and this sequence (bearing K112) appears to be a lab-generated transconjugant of COL (strain COLVA/NRS281)[58]. Based on mutational and structural analysis of Prs from *Escherichia coli* and *Bacillus subtilis* (which shares 77% amino acid identity with the *S. aureus* enzyme), E112 has not previously been identified as a residue important for substrate binding, intramolecular interactions or allosteric regulation[59,60]. However, it is found directly adjacent to an allosteric binding site. Without a detailed enzymological investigation, we can only speculate on the effect the E112K mutation has on the activity of Prs. Nevertheless, given the slow growth phenotype of COL and the downregulation of IMP, histidine and tryptophan synthesis genes detected in our transcriptomic analysis, it seems most likely that the E112K mutation has negatively impacted the PRPP synthesis activity of Prs.

To our knowledge, COL represents the first reported case of a Prs mutation in a clinical isolate. However, other Prs mutations have been reported in *in vitro*-evolved tolerant strains of *S. aureus* and *E. coli* under antibiotic pressure[41,52,61], indicating that dysregulation of PRPP synthesis may not be an uncommon mechanism of genotypic tolerance. In general, there are conflicting data in the literature regarding the relationship between purine synthesis, tolerance and persistent infections[43,62–64] which requires further investigation.

COL is a commonly used model strain of MRSA in both *in vitro* and *in vivo* studies of antibiotic efficacy[14–16,18–21]. Therefore, identification of its tolerant phenotype is highly relevant and indicates that COL is an atypical strain of MRSA. While COL represents an invaluable and clinically relevant example of tolerant *S. aureus* for much-needed wider studies of tolerance (*e.g.* animal models of antibiotic efficacy, impact of tolerance on

development of resistance)[38], its unusual growth and tolerance phenotypes should be considered when using it as a model MRSA strain in other studies. Overall, identification of tolerance in COL, which was isolated in the 1960s[8], shows that antibiotic tolerance in this strain predates the seminal first report of tolerance among clinical isolates by more than 10 years[37]. It also indicates that clinical antibiotic tolerance occurred in *S. aureus* around the same time as, or even before, clinical methicillin resistance. The clinical prevalence and significance of antibiotic tolerance, as well as its ability to promote the development of endogenous resistance, remains a largely unexplored area of clinical microbiology that requires attention.

Experimental Procedures

Antibiotics and reagents

X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) and chloramphenicol were purchased from Bio Basic Inc. (Markham, ON, Canada). Daptomycin and ppGpp were obtained from Toronto Research Chemicals (Toronto, ON, Canada) and Jena Bioscience (Jena, Germany), respectively. All other antibiotics, enzymes, and reagents, unless otherwise stated, were from MilliporeSigma (Gillingham, UK).

Bacterial strains, plasmids and growth conditions

S. aureus strains Newman and MN8 were gifts from Michael Murphy (University of British Columbia) and Mariya Goncheva (University of Victoria, British Columbia), respectively, while strains COL, N315, MW2 and LAC were gifts from Paul Kubes (University of Calgary). COL *rpoB*$^{+}$ was a gift from Simon Foster (University of Sheffield). The Newman *rel* F128Y mutant has been reported previously[33]. *Escherichia coli* Stellar (TaKaRa Bio USA, Mountain View, CA) was used for all cloning, while *E. coli* IM08B[65] was used to

prepare methylated plasmid for transformation into Newman and COL. Plasmid pIMAY-Z[65] was a gift from Ian Monk (University of Melbourne, Australia). *S. aureus* strains were routinely cultured in tryptic soy broth/tryptic soy agar (TSB/TSA) at 37°C and stored long term at −80°C with 8% (vol/vol) glycerol.

Allelic exchange

Allelic exchange of *prs* (NWMN_RS02645, SACOL0544) and *gltX* (NWMN_RS02865, SACOL0574) was performed using pIMAY-Z as previously described[33]. For allelic exchange in Newman, both genes were amplified from Newman genomic DNA as two halves using CloneAmp HiFi PCR premix (TaKaRa Bio) and the COL mutation-containing primers listed in Table S1 (E112K for *prs* and E405K for *gltX*). The two halves were then joined together by overlap extension PCR and cloned into pIMAY-Z between the *Eco*RI and *Not*I sites using In-Fusion Snap Assembly mastermix (TaKaRa Bio). To generate the constructs for allelic exchange in COL, *prs* was amplified from COL genomic DNA as two halves with mutagenic primers (K112E) as above, while the Newman *gltX* pIMAY-Z construct was mutated to K405E *via* site-directed mutagenesis. Following construct confirmation by bidirectional sequencing and passage through IM08B for methylation, constructs were introduced into Newman by electroporation and COL by phage transduction with ϕ85[66]. pIMAY-Z integration and excision was performed as previously described[33]. Primers that bind outside of the mutated gene were used to amplify the region for confirmatory bidirectional sequencing (Table S1).

Growth curve analysis

Growth curves for all strains were performed in quadruplicate in 5 mL of TSB in 16 mm test tubes. Overnight cultures derived from different colonies were diluted (10 uL in 5 mL

fresh TSB) and grown at 37°C with 200 rpm shaking. OD_{600nm} was recorded using a test tube spectrophotometer every 30 minutes for the first 90 minutes, and then every 15 minutes until an OD_{600nm} of 2.0 was reached. Specific growth rates (μ) were determined from the slope of $\ln(OD_{600nm})$ versus time during exponential phase, and converted into doubling times using the equation $\ln(2)/\mu$. Lag times were found by extrapolating the slope of the exponential phase back to the initial OD_{600nm}. All statistical analysis was performed in GraphPad Prism 6.07.

Minimum inhibitory concentration (MIC) determinations

Strains of interest were grown overnight in 5 mL of Mueller-Hinton broth (MHB) at 37°C with 200 rpm shaking. MIC determinations were performed in triplicate using the broth microdilution method of the Clinical and Laboratory Standards Institute (CLSI)[67] in MHB or MHB supplemented with 50 μg/mL Ca^{2+} (MHB + Ca^{2+}) for daptomycin.

Time-kill assays

Time-kill assays were performed in triplicate in MHB or MHB + Ca^{+2} in 18 mm test tubes at 37°C according to CLSI guidelines[68]. Antibiotics were tested at 8 × MIC for daptomycin and 16 × MIC for ciprofloxacin for all strains. Aliquots were taken from growing cultures at defined time points, diluted in phosphate-buffered saline and plated in duplicate on TSA agar. Colony forming units (CFU) were counted using the aCOLyte 3 HD automated colony counter (Synbiosis Ltd, Cambridge), with counts periodically confirmed manually. The limit of detection following antibiotic dilution was 300 CFU/mL. MDK_{99} values were calculated using linear regression and data interpolation in GraphPad Prism 6.07; only

data in the linear range of the MDK were used in the calculation (a minimum of three data points) and all R^2 values were >0.9.

(p)ppGpp quantitation by HPLC

(p)ppGpp extraction and quantitation was performed largely as described by Varik et al[55]. Quadruplicate cultures of 250 mL in TSB were inoculated with 5 mL of overnight culture (originating from different colonies), grown at 37°C with shaking to an OD_{600nm} of 0.6-0.8 and filtered through 45 µm cellulose acetate filters (6 per sample) using a vacuum manifold (MilliporeSigma). Filters were immediately added to a 15 mL falcon tube containing 5 mL 1 M acetic acid, then flash frozen in liquid nitrogen and stored at -80°C. To extract nucleotides, samples were thawed on ice then vortexed for 5-10 seconds, every 5 minutes, for 30 minutes and kept on ice in between. Filters were removed and the extract flash frozen in liquid nitrogen and stored at -80°C. Samples were freeze-dried overnight, resuspended in 600 µL of milliQ water, centrifuged to remove cell debris, and the supernatant stored at -20°C until analysis. Nucleotides were separated *via* strong-anion exchange (SAX) chromatography using a SphereClone SAX (4.6 × 150 mm, 5 µm) column fitted with a SecurityGuard cartridge (Phenomenex Ltd, Macclesfield, UK) run on a Shimadzu HPLC system (UV detection: 252 nm) using a 32 minute isocratic program with 0.36 M $NH_4H_2PO_4$ pH 3.4, 25 % (v/v) acetonitrile and a flow rate of 0.5 mL/min. Elution time and peaks corresponding to ppGpp were identified by spiking every 8th run with 2 mM ppGpp (Jena Bioscience). Area under the peak for ppGpp was calculated using LabSolutions software (Shimadzu) and corrected for the OD_{600nm} of the culture at harvest.

RNA sequencing

RNA was extracted from 2 mL of quadruplicate cultures grown in TSB to OD_{600nm} ~0.6. Cultures were combined with 2 volumes of RNAprotect® (Qiagen, Manchester, UK) and incubated for 5 mins at room temperature, then centrifuged at 5000 rpm for 10 mins. The supernatant was removed and frozen at -20°C until extraction. RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer's instructions using both mechanical disruption and chemical disruption with lysostaphin. Following extraction, the samples were treated with DNase and incubated at 37°C for 30 mins to eliminate contamination with DNA. RNA was then repurified using the same protocol and column from the RNeasy kit. Quality and concentration of samples was determined using Nanodrop ND-1000 and Qubit readings with the RNA BR Assay kit. RNA samples were sequenced by Novogene (Cambridge, UK) on an illumina NovaSeq 6000 using a 150 bp paired-end protocol. Gene expression was quantified using Salmon v1.10.2[69] by pseudoaligning reads to CDS from NC_009641.1[70]. The resulting counts were modelled as a function of strain using DESeq2 1.40.2[71]. Genes lacking orthologues in COL were identified using AureoWiki[72] and removed from the analysis. Differential gene expression was determined by a threshold of absolute fold change greater than 2 and an FDR-adjusted *p*-value less than 0.05. Hypergeometric tests (one-tailed Fisher's exact tests) were used to identify over-represented biological process gene ontology terms using ShinyGO v0.81[73]. Raw sequencing data has been deposited under BioProject accession number PRJNA1196949.

Bioinformatic analysis of Prs

A search of GenBank with "ribose-phosphate pyrophosphokinase" and "*Staphylococcus aureus*" and a custom sequence length of 275 to 330 amino acids resulted in 10,571 protein entries. ElimDupes (https://www.hiv.lanl.gov/content/sequence/elimdupesv2/elimdupes.html) was used to remove identical sequences, resulting in 59 unique entries.

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References

(1) Tong, S. Y. C.; Davis, J. S.; Eichenberger, E.; Holland, T. L.; Fowler, V. G. *Staphylococcus aureus* Infections: Epidemiology, Pathophysiology, Clinical Manifestations, and Management. *Clin. Microbiol. Rev.* **2015**, *28* (3), 603–661.

(2) Harkins, C. P.; Pichon, B.; Doumith, M.; Parkhill, J.; Westh, H.; Tomasz, A.; de Lencastre, H.; Bentley, S. D.; Kearns, A. M.; Holden, M. T. G. Methicillin-Resistant *Staphylococcus aureus* Emerged Long before the Introduction of Methicillin into Clinical Practice. *Genome Biol.* **2017**, *18* (1), 130.

(3) Murray, C. J.; Ikuta, K. S.; Sharara, F.; Swetschinski, L.; Aguilar, G. R.; Gray, A.; Han, C.; Bisignano, C.; Rao, P.; Wool, E.; Johnson, S. C.; Browne, A. J.; Chipeta, M. G.; Fell, F.; Hackett, S.; Haines-Woodhouse, G.; Hamadani, B. H. K.; Kumaran, E. A. P.; McManigal, B.; Agarwal, R.; Akech, S.; Albertson, S.; Amuasi, J.; Andrews, J.; Aravkin, A.; Ashley, E.; Bailey, F.; Baker, S.; Basnyat, B.; Bekker, A.; Bender, R.; Bethou, A.; Bielicki, J.; Boonkasidecha, S.; Bukosia, J.; Carvalheiro, C.; Castañeda-Orjuela, C.; Chansamouth, V.; Chaurasia, S.; Chiurchiù, S.; Chowdhury, F.; Cook, A. J.; Cooper, B.; Cressey, T. R.; Criollo-Mora, E.; Cunningham, M.; Darboe, S.; Day, N. P. J.; Luca, M. D.; Dokova, K.; Dramowski, A.; Dunachie, S. J.; Eckmanns, T.; Eibach, D.; Emami, A.; Feasey, N.; Fisher-Pearson, N.; Forrest, K.; Garrett, D.;

Gastmeier, P.; Giref, A. Z.; Greer, R. C.; Gupta, V.; Haller, S.; Haselbeck, A.; Hay, S. I.; Holm, M.; Hopkins, S.; Iregbu, K. C.; Jacobs, J.; Jarovsky, D.; Javanmardi, F.; Khorana, M.; Kissoon, N.; Kobeissi, E.; Kostyanev, T.; Krapp, F.; Krumkamp, R.; Kumar, A.; Kyu, H. H.; Lim, C.; Limmathurotsakul, D.; Loftus, M. J.; Lunn, M.; Ma, J.; Mturi, N.; Munera-Huertas, T.; Musicha, P.; Mussi-Pinhata, M. M.; Nakamura, T.; Nanavati, R.; Nangia, S.; Newton, P.; Ngoun, C.; Novotney, A.; Nwakanma, D.; Obiero, C. W.; Olivas-Martinez, A.; Olliaro, P.; Ooko, E.; Ortiz-Brizuela, E.; Peleg, A. Y.; Perrone, C.; Plakkal, N.; Ponce-de-Leon, A.; Raad, M.; Ramdin, T.; Riddell, A.; Roberts, T.; Robotham, J. V.; Roca, A.; Rudd, K. E.; Russell, N.; Schnall, J.; Scott, J. A. G.; Shivamallappa, M.; Sifuentes-Osornio, J.; Steenkeste, N.; Stewardson, A. J.; Stoeva, T.; Tasak, N.; Thaiprakong, A.; Thwaites, G.; Turner, C.; Turner, P.; Doorn, H. R. van; Velaphi, S.; Vongpradith, A.; Vu, H.; Walsh, T.; Waner, S.; Wangrangsimakul, T.; Wozniak, T.; Zheng, P.; Sartorius, B.; Lopez, A. D.; Stergachis, A.; Moore, C.; Dolecek, C.; Naghavi, M. Global Burden of Bacterial Antimicrobial Resistance in 2019: A Systematic Analysis. *Lancet* **2022**, *399* (10325), 629–655.

(4) Miragaia, M. Factors Contributing to the Evolution of *mecA*-Mediated β-Lactam Resistance in Staphylococci: Update and New Insights From Whole Genome Sequencing (WGS). *Front. Microbiol.* **2018**, *9*, 2723.

(5) Bilyk, B. L.; Panchal, V. V.; Tinajero-Trejo, M.; Hobbs, J. K.; Foster, S. J. An Interplay of Multiple Positive and Negative Factors Governs Methicillin Resistance in *Staphylococcus aureus. Microbiol. Mol. Biol. Rev. MMBR* **2022**, *86* (2), e0015921.

(6) Tomasz, A.; Nachman, S.; Leaf, H. Stable Classes of Phenotypic Expression in Methicillin-Resistant Clinical Isolates of Staphylococci. *Antimicrob. Agents Chemother.* **1991**, *35* (1), 124–129.

(7) De Lencastre, H.; Wu, S. W.; Pinho, M. G.; Ludovice, A. M.; Filipe, S.; Gardete, S.; Sobral, R.; Gill, S.; Chung, M.; Tomasz, A. Antibiotic Resistance as a Stress Response: Complete Sequencing of a Large Number of Chromosomal Loci in *Staphylococcus aureus* Strain COL That Impact on the Expression of Resistance to Methicillin. *Microb. Drug Resist.* **1999**, *5* (3), 163–175.

(8) Dyke, K. G.; Jevons, M. P.; Parker, M. T. Penicillinase Production and Intrinsic Resistance to Penicillins in *Staphylococcus aureus. Lancet* **1966**, *1* (7442), 835–838.

(9) Sabath, L. D.; Wallace, S. J.; Gerstein, D. A. Suppression of Intrinsic Resistance to Methicillin and Other Penicillins in *Staphylococcus aureus. Antimicrob. Agents Chemother.* **1972**, *2* (5), 350–355.

(10) Wilkinson, B. J.; Dorian, K. J.; Sabath, L. D. Cell Wall Composition and Associated Properties of Methicillin-Resistant *Staphylococcus aureus* Strains. *J. Bacteriol.* **1978**, *136* (3), 976–982.

(11) Chambers, H. F.; DeLeo, F. R. Waves of Resistance: *Staphylococcus aureus* in the Antibiotic Era. *Nat. Rev. Microbiol.* **2009**, *7* (9), 629–641.

(12) Bowers, J. R.; Driebe, E. M.; Albrecht, V.; McDougal, L. K.; Granade, M.; Roe, C. C.; Lemmer, D.; Rasheed, J. K.; Engelthaler, D. M.; Keim, P.; Limbago, B. M. Improved Subtyping of *Staphylococcus aureus* Clonal Complex 8 Strains Based on Whole-Genome Phylogenetic Analysis. *mSphere* **2018**, *3* (3), e00464-17.

(13) Reed, P.; Atilano, M. L.; Alves, R.; Hoiczyk, E.; Sher, X.; Reichmann, N. T.; Pereira, P. M.; Roemer, T.; Filipe, S. R.; Pereira-Leal, J. B.; Ligoxygakis, P.; Pinho, M. G.

Staphylococcus aureus Survives with a Minimal Peptidoglycan Synthesis Machine but Sacrifices Virulence and Antibiotic Resistance. *PLoS Pathog.* **2015**, *11* (5), e1004891.
(14) Xiao, Q.; Vakulenko, S.; Chang, M.; Mobashery, S. Mutations in *mmpL* and in the Cell Wall Stress Stimulon Contribute to Resistance to Oxadiazole Antibiotics in Methicillin-Resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **2014**, *58* (10), 5841–5847.
(15) Lama, A.; Pané-Farré, J.; Chon, T.; Wiersma, A. M.; Sit, C. S.; Vederas, J. C.; Hecker, M.; Nakano, M. M. Response of Methicillin-Resistant *Staphylococcus aureus* to Amicoumacin A. *PLoS One* **2012**, *7* (3), e34037.
(16) Yeo, W.-S.; Jeong, B.; Ullah, N.; Shah, M. A.; Ali, A.; Kim, K. K.; Bae, T. Ftsh Sensitizes Methicillin-Resistant *Staphylococcus aureus* to β-Lactam Antibiotics by Degrading YpfP, a Lipoteichoic Acid Synthesis Enzyme. *Antibiot. Basel Switz.* **2021**, *10* (10), 1198.
(17) Tuffs, S. W.; Goncheva, M. I.; Xu, S. X.; Craig, H. C.; Kasper, K. J.; Choi, J.; Flannagan, R. S.; Kerfoot, S. M.; Heinrichs, D. E.; McCormick, J. K. Superantigens Promote *Staphylococcus aureus* Bloodstream Infection by Eliciting Pathogenic Interferon-Gamma Production. *Proc. Natl. Acad. Sci.* **2022**, *119* (8), e2115987119.
(18) Surewaard, B. G. J.; Deniset, J. F.; Zemp, F. J.; Amrein, M.; Otto, M.; Conly, J.; Omri, A.; Yates, R. M.; Kubes, P. Identification and Treatment of the *Staphylococcus aureus* Reservoir *in vivo. J. Exp. Med.* **2016**, *213* (7), 1141–1151.
(19) Tattevin, P.; Basuino, L.; Bauer, D.; Diep, B. A.; Chambers, H. F. Ceftobiprole Is Superior to Vancomycin, Daptomycin, and Linezolid for Treatment of Experimental Endocarditis in Rabbits Caused by Methicillin-Resistant *Staphylococcus aureus. Antimicrob. Agents Chemother.* **2010**, *54* (2), 610–613.
(20) Goetz, J.; Keyssner, V.; Hanses, F.; Greimel, F.; Leiß, F.; Schwarz, T.; Springorum, H.-R.; Grifka, J.; Schaumburger, J. Animal Experimental Investigation on the Efficacy of Antibiotic Therapy with Linezolid, Vancomycin, Cotrimoxazole, and Rifampin in Treatment of Periprosthetic Knee Joint Infections by MRSA. *Bone Jt. Res.* **2022**, *11* (3), 143–151.
(21) Madrigal, A. G.; Basuino, L.; Chambers, H. F. Efficacy of Telavancin in a Rabbit Model of Aortic Valve Endocarditis Due to Methicillin-Resistant *Staphylococcus aureus* or Vancomycin-Intermediate *Staphylococcus aureus. Antimicrob. Agents Chemother.* **2005**, *49* (8), 3163–3165.
(22) Li, M.; Diep, B. A.; Villaruz, A. E.; Braughton, K. R.; Jiang, X.; DeLeo, F. R.; Chambers, H. F.; Lu, Y.; Otto, M. Evolution of Virulence in Epidemic Community-Associated Methicillin-Resistant *Staphylococcus aureus. Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (14), 5883–5888.
(23) Kim, C. K.; Milheiriço, C.; de Lencastre, H.; Tomasz, A. Antibiotic Resistance as a Stress Response: Recovery of High-Level Oxacillin Resistance in Methicillin-Resistant *Staphylococcus aureus* “Auxiliary” (Fem) Mutants by Induction of the Stringent Stress Response. *Antimicrob. Agents Chemother.* **2017**, *61* (8), e00313-17.
(24) Irving, S. E.; Choudhury, N. R.; Corrigan, R. M. The Stringent Response and Physiological Roles of (pp)pGpp in Bacteria. *Nat. Rev. Microbiol.* **2021**, *19* (4), 256–271.

(25) Atkinson, G. C.; Tenson, T.; Hauryliuk, V. The RelA/SpoT Homolog (RSH) Superfamily: Distribution and Functional Evolution of ppGpp Synthetases and Hydrolases across the Tree of Life. *PloS One* **2011**, *6* (8), e23479.
(26) Hauryliuk, V.; Atkinson, G. C.; Murakami, K. S.; Tenson, T.; Gerdes, K. Recent Functional Insights into the Role of (p)ppGpp in Bacterial Physiology. *Nat. Rev. Microbiol.* **2015**, *13* (5), 298–309.
(27) Geiger, T.; Wolz, C. Intersection of the Stringent Response and the CodY Regulon in Low GC Gram-Positive Bacteria. *Int. J. Med. Microbiol. IJMM* **2014**, *304* (2), 150–155.
(28) Krásný, L.; Tišerová, H.; Jonák, J.; Rejman, D.; Šanderová, H. The Identity of the Transcription +1 Position Is Crucial for Changes in Gene Expression in Response to Amino Acid Starvation in *Bacillus subtilis. Mol. Microbiol.* **2008**, *69* (1), 42–54.
(29) Corrigan, R. M.; Bellows, L. E.; Wood, A.; Gründling, A. ppGpp Negatively Impacts Ribosome Assembly Affecting Growth and Antimicrobial Tolerance in Gram-Positive Bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113* (12), E1710-1719.
(30) Steinchen, W.; Zegarra, V.; Bange, G. (p)ppGpp: Magic Modulators of Bacterial Physiology and Metabolism. *Front. Microbiol.* **2020**, *11*, 2072.
(31) Kriel, A.; Bittner, A. N.; Kim, S. H.; Liu, K.; Tehranchi, A. K.; Zou, W. Y.; Rendon, S.; Chen, R.; Tu, B. P.; Wang, J. D. Direct Regulation of GTP Homeostasis by (p)ppGpp: A Critical Component of Viability and Stress Resistance. *Mol. Cell* **2012**, *48* (2), 231–241.
(32) Salzer, A.; Wolz, C. Role of (p)ppGpp in Antibiotic Resistance, Tolerance, Persistence and Survival in Firmicutes. *microLife* **2023**, *4*, uqad009.
(33) Bryson, D.; Hettle, A. G.; Boraston, A. B.; Hobbs, J. K. Clinical Mutations That Partially Activate the Stringent Response Confer Multidrug Tolerance in Staphylococcus aureus. *Antimicrob. Agents Chemother.* **2020**, *64* (3), e02103-19.
(34) Deventer, A. T.; Bryson, D.; Shortill, M.; Boraston, A. B.; Hobbs, J. K. Molecular Characterization of Clinical Rel Mutations and Consequences for Resistance Expression and Fitness in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **2022**, *66* (12), e0093822.
(35) Brauner, A.; Fridman, O.; Gefen, O.; Balaban, N. Q. Distinguishing between Resistance, Tolerance and Persistence to Antibiotic Treatment. *Nat. Rev. Microbiol.* **2016**, *14* (5), 320–330.
(36) Tomasz, A.; Albino, A.; Zanati, E. Multiple Antibiotic Resistance in a Bacterium with Suppressed Autolytic System. *Nature* **1970**, *227* (5254), 138–140.
(37) Sabath, L. D.; Wheeler, N.; Laverdiere, M.; Blazevic, D.; Wilkinson, B. J. A New Type of Penicillin Resistance of *Staphylococcus aureus. Lancet* **1977**, *1* (8009), 443–447.
(38) Deventer, A. T.; Stevens, C. E.; Stewart, A.; Hobbs, J. K. Antibiotic Tolerance among Clinical Isolates: Mechanisms, Detection, Prevalence and Significance. *Clin. Microbiol. Rev.* **2024**, *37* (4), e0010624.
(39) Kuehl, R.; Morata, L.; Meylan, S.; Mensa, J.; Soriano, A. When Antibiotics Fail: A Clinical and Microbiological Perspective on Antibiotic Tolerance and Persistence of *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **2020**, *75* (5), 1071–1086.
(40) Lazarovits, G.; Gefen, O.; Cahanian, N.; Adler, K.; Fluss, R.; Levin-Reisman, I.; Ronin, I.; Motro, Y.; Moran-Gilad, J.; Balaban, N. Q.; Strahilevitz, J. Prevalence of

Antibiotic Tolerance and Risk for Reinfection among *Escherichia coli* Bloodstream Isolates: A Prospective Cohort Study. *Clin. Infect. Dis.* **2022**, *75* (10), 1706–1713.
(41) Levin-Reisman, I.; Ronin, I.; Gefen, O.; Braniss, I.; Shoresh, N.; Balaban, N. Q. Antibiotic Tolerance Facilitates the Evolution of Resistance. *Science* **2017**, *355* (6327), 826–830.
(42) Santi, I.; Manfredi, P.; Maffei, E.; Egli, A.; Jenal, U. Evolution of Antibiotic Tolerance Shapes Resistance Development in Chronic *Pseudomonas aeruginosa* Infections. *mBio* **2021**, *12* (1), e03482-20.
(43) Liu, J.; Gefen, O.; Ronin, I.; Bar-Meir, M.; Balaban, N. Q. Effect of Tolerance on the Evolution of Antibiotic Resistance under Drug Combinations. *Science* **2020**, *367* (6474), 200–204.
(44) Proctor, R. A.; Kriegeskorte, A.; Kahl, B. C.; Becker, K.; Löffler, B.; Peters, G. *Staphylococcus aureus* Small Colony Variants (SCVs): A Road Map for the Metabolic Pathways Involved in Persistent Infections. *Front. Cell. Infect. Microbiol.* **2014**, *4*, 99.
(45) Stokes, J. M.; Lopatkin, A. J.; Lobritz, M. A.; Collins, J. J. Bacterial Metabolism and Antibiotic Efficacy. *Cell Metab.* **2019**, *30* (2), 251–259.
(46) Hobbs, J. K.; Boraston, A. B. (p)ppGpp and the Stringent Response: An Emerging Threat to Antibiotic Therapy. *ACS Infect. Dis.* **2019**, *5* (9), 1505–1517.
(47) Tuomanen, E.; Cozens, R.; Tosch, W.; Zak, O.; Tomasz, A. The Rate of Killing of *Escherichia coli* by Beta-Lactam Antibiotics Is Strictly Proportional to the Rate of Bacterial Growth. *J. Gen. Microbiol.* **1986**, *132* (5), 1297–1304.
(48) Lopatkin, A. J.; Stokes, J. M.; Zheng, E. J.; Yang, J. H.; Takahashi, M. K.; You, L.; Collins, J. J. Bacterial Metabolic State More Accurately Predicts Antibiotic Lethality than Growth Rate. *Nat. Microbiol.* **2019**, *4* (12), 2109–2117.
(49) Lee, A. J.; Wang, S.; Meredith, H. R.; Zhuang, B.; Dai, Z.; You, L. Robust, Linear Correlations between Growth Rates and β-Lactam–Mediated Lysis Rates. *Proc. Natl. Acad. Sci.* **2018**, *115* (16), 4069–4074.
(50) Bren, A.; Glass, D. S.; Kohanim, Y. K.; Mayo, A.; Alon, U. Tradeoffs in Bacterial Physiology Determine the Efficiency of Antibiotic Killing. *Proc. Natl. Acad. Sci.* **2023**, *120* (51), e2312651120.
(51) Lobritz, M. A.; Belenky, P.; Porter, C. B. M.; Gutierrez, A.; Yang, J. H.; Schwarz, E. G.; Dwyer, D. J.; Khalil, A. S.; Collins, J. J. Antibiotic Efficacy Is Linked to Bacterial Cellular Respiration. *Proc. Natl. Acad. Sci.* **2015**, *112* (27), 8173–8180.
(52) Fridman, O.; Goldberg, A.; Ronin, I.; Shoresh, N.; Balaban, N. Q. Optimization of Lag Time Underlies Antibiotic Tolerance in Evolved Bacterial Populations. *Nature* **2014**, *513* (7518), 418–421.
(53) Panchal, V. V.; Griffiths, C.; Mosaei, H.; Bilyk, B.; Sutton, J. A. F.; Carnell, O. T.; Hornby, D. P.; Green, J.; Hobbs, J. K.; Kelley, W. L.; Zenkin, N.; Foster, S. J. Evolving MRSA: High-Level β-Lactam Resistance in *Staphylococcus aureus* Is Associated with RNA Polymerase Alterations and Fine Tuning of Gene Expression. *PLoS Pathog.* **2020**, *16* (7), e1008672.
(54) Vinella, D.; D'Ari, R.; Jaffé, A.; Bouloc, P. Penicillin Binding Protein 2 Is Dispensable in *Escherichia coli* When ppGpp Synthesis Is Induced. *EMBO J.* **1992**, *11* (4), 1493–1501.

(55) Varik, V.; Oliveira, S. R. A.; Hauryliuk, V.; Tenson, T. HPLC-Based Quantification of Bacterial Housekeeping Nucleotides and Alarmone Messengers ppGpp and pppGpp. *Sci. Rep.* **2017**, *7* (1), 11022.
(56) Geiger, T.; Francois, P.; Liebeke, M.; Fraunholz, M.; Goerke, C.; Krismer, B.; Schrenzel, J.; Lalk, M.; Wolz, C. The Stringent Response of *Staphylococcus aureus* and Its Impact on Survival after Phagocytosis through the Induction of Intracellular PSMs Expression. *PLoS Pathog.* **2012**, *8* (11), e1003016.
(57) Hove-Jensen, B.; Andersen, K. R.; Kilstrup, M.; Martinussen, J.; Switzer, R. L.; Willemoës, M. Phosphoribosyl Diphosphate (PRPP): Biosynthesis, Enzymology, Utilization, and Metabolic Significance. *Microbiol. Mol. Biol. Rev. MMBR* **2017**, *81* (1), e00040-16.
(58) Weigel, L. M.; Clewell, D. B.; Gill, S. R.; Clark, N. C.; McDougal, L. K.; Flannagan, S. E.; Kolonay, J. F.; Shetty, J.; Killgore, G. E.; Tenover, F. C. Genetic Analysis of a High-Level Vancomycin-Resistant Isolate of *Staphylococcus aureus. Science* **2003**, *302* (5650), 1569–1571.
(59) Eriksen, T. A.; Kadziola, A.; Bentsen, A. K.; Harlow, K. W.; Larsen, S. Structural Basis for the Function of *Bacillus aubtilis* Phosphoribosyl-Pyrophosphate Synthetase. *Nat. Struct. Biol.* **2000**, *7* (4), 303–308.
(60) Zhou, W.; Tsai, A.; Dattmore, D. A.; Stives, D. P.; Chitrakar, I.; D'alessandro, A. M.; Patil, S.; Hicks, K. A.; French, J. B. Crystal Structure of *E. coli* PRPP Synthetase. *BMC Struct. Biol.* **2019**, *19*, 1.
(61) Sulaiman, J. E.; Lam, H. Novel Daptomycin Tolerance and Resistance Mutations in Methicillin-Resistant *Staphylococcus aureus* from Adaptive Laboratory Evolution. *mSphere* **2021**, *6* (5), e0069221.
(62) Xiong, Y. Q.; Li, Y.; Goncheva, M. I.; Elsayed, A. M.; Zhu, F.; Li, L.; Abdelhady, W.; Flannagan, R. S.; Yeaman, M. R.; Bayer, A. S.; Heinrichs, D. E. The Purine Biosynthesis Repressor, PurR, Contributes to Vancomycin Susceptibility of Methicillin-Resistant *Staphylococcus aureus* in Experimental Endocarditis. *J. Infect. Dis.* **2024**, *229* (6), 1648-1657.
(63) Yang, J. H.; Wright, S. N.; Hamblin, M.; McCloskey, D.; Alcantar, M. A.; Schrübbers, L.; Lopatkin, A. J.; Satish, S.; Nili, A.; Palsson, B. O.; Walker, G. C.; Collins, J. J. A White-Box Machine Learning Approach for Revealing Antibiotic Mechanisms of Action. *Cell* **2019**, *177* (6), 1649-1661.e9.
(64) Li, L.; Abdelhady, W.; Donegan, N. P.; Seidl, K.; Cheung, A.; Zhou, Y.-F.; Yeaman, M. R.; Bayer, A. S.; Xiong, Y. Q. Role of Purine Biosynthesis in Persistent Methicillin-Resistant *Staphylococcus aureus* Infection. *J. Infect. Dis.* **2018**, *218* (9), 1367–1377.
(65) Monk, I. R.; Tree, J. J.; Howden, B. P.; Stinear, T. P.; Foster, T. J. Complete Bypass of Restriction Systems for Major *Staphylococcus aureus* Lineages. *mBio* **2015**, *6* (3), 10.1128/mbio.00308-15.
(66) Olson, M. E. Bacteriophage Transduction in *Staphylococcus aureus. Methods Mol. Biol.* **2016**, *1373*, 69–74.
(67) Clinical and Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 12th Edition. **2018**.
(68) Clinical and Laboratory Standards Institute. Methods for Determining Bactericidal Activity of Antimicrobial Agents: Approved Guideline M26-A. **1999**.

(69) Patro, R.; Duggal, G.; Love, M. I.; Irizarry, R. A.; Kingsford, C. Salmon Provides Fast and Bias-Aware Quantification of Transcript Expression. *Nat. Methods* **2017**, *14* (4), 417–419.
(70) Baba, T.; Bae, T.; Schneewind, O.; Takeuchi, F.; Hiramatsu, K. Genome Sequence of *Staphylococcus aureus* Strain Newman and Comparative Analysis of Staphylococcal Genomes: Polymorphism and Evolution of Two Major Pathogenicity Islands. *J. Bacteriol.* **2008**, *190* (1), 300–310.
(71) Love, M. I.; Huber, W.; Anders, S. Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2. *Genome Biol.* **2014**, *15* (12), 550.
(72) Fuchs, S.; Mehlan, H.; Bernhardt, J.; Hennig, A.; Michalik, S.; Surmann, K.; Pané-Farré, J.; Giese, A.; Weiss, S.; Backert, L.; Herbig, A.; Nieselt, K.; Hecker, M.; Völker, U.; Mäder, U. AureoWiki - The Repository of the *Staphylococcus aureus* Research and Annotation Community. *Int. J. Med. Microbiol. IJMM* **2018**, *308* (6), 558–568.
(73) Ge, S. X.; Jung, D.; Yao, R. ShinyGO: A Graphical Gene-Set Enrichment Tool for Animals and Plants. *Bioinforma.* **2020**, *36* (8), 2628–2629.
(74) Duthie, E. S.; Lorenz, L. L. Staphylococcal Coagulase: Mode of Action and Antigenicity. *Microbiology* **1952**, *6* (1–2), 95–107.
(75) Voyich, J. M.; Braughton, K. R.; Sturdevant, D. E.; Whitney, A. R.; Saïd-Salim, B.; Porcella, S. F.; Long, R. D.; Dorward, D. W.; Gardner, D. J.; Kreiswirth, B. N.; Musser, J. M.; DeLeo, F. R. Insights into Mechanisms Used by *Staphylococcus aureus* to Avoid Destruction by Human Neutrophils1. *J. Immunol.* **2005**, *175* (6), 3907–3919.
(76) Baba, T.; Takeuchi, F.; Kuroda, M.; Yuzawa, H.; Aoki, K.; Oguchi, A.; Nagai, Y.; Iwama, N.; Asano, K.; Naimi, T.; Kuroda, H.; Cui, L.; Yamamoto, K.; Hiramatsu, K. Genome and Virulence Determinants of High Virulence Community-Acquired MRSA. *Lancet* **2002**, *359* (9320), 1819–1827.
(77) Kuroda, M.; Ohta, T.; Uchiyama, I.; Baba, T.; Yuzawa, H.; Kobayashi, I.; Cui, L.; Oguchi, A.; Aoki, K.; Nagai, Y.; Lian, J.; Ito, T.; Kanamori, M.; Matsumaru, H.; Maruyama, A.; Murakami, H.; Hosoyama, A.; Mizutani-Ui, Y.; Takahashi, N. K.; Sawano, T.; Inoue, R.; Kaito, C.; Sekimizu, K.; Hirakawa, H.; Kuhara, S.; Goto, S.; Yabuzaki, J.; Kanehisa, M.; Yamashita, A.; Oshima, K.; Furuya, K.; Yoshino, C.; Shiba, T.; Hattori, M.; Ogasawara, N.; Hayashi, H.; Hiramatsu, K. Whole Genome Sequencing of Meticillin-Resistant *Staphylococcus aureus. Lancet* **2001**, *357* (9264), 1225–1240.
(78) Schlievert, P. M.; Blomster, D. A. Production of Staphylococcal Pyrogenic Exotoxin Type C: Influence of Physical and Chemical Factors. *J. Infect. Dis.* **1983**, *147* (2), 236–242.

Table 1. Model strains of *S. aureus* used in this study and MIC values

Strain name	Lineage	Clonal complex	Sequence type	Description	Genome accession number	Reference	MIC (mg/L)[a]	
							Ciprofloxacin	Daptomycin
COL	Archaic	CC8	ST250	HA-MRSA	NC_002951	[8]	0.125	0.5
Newman	Archaic	CC8	ST8	MSSA	NC_009641	[74]	0.125	0.5
LAC	USA300	CC8	ST8	CA-MRSA	CP035369	[75]	>8	0.5
MW2	USA400	CC1	ST1	CA-MRSA	NC_003923	[76]	0.125	1
N315	-	CC5	ST5	HA-MRSA	BA000018	[77]	0.25	1
MN8	USA200	CC30	ST30	MSSA	CP091875	[78]	0.25	0.5

[a]MIC values were unchanged between wildtype and mutant variants of COL and Newman
Abbreviations: hospital-acquired (HA), community-acquired (CA), methicillin-sensitive *S. aureus* (MSSA).

Table 2. Lag times, doubling times and MDK_{99} values for wildtype COL, wildtype Newman, and COL single, double and triple mutant strains[a]

Strain	Lag time (mins)	Doubling time (mins)	MDK_{99} (CIP)	MDK_{99} (DAP)
COL	155.7 ± 3.5	41.6 ± 1.7	3.9 ± 0.2	2.1 ± 0.1
Newman	115.3 ± 5.4 (74%)	37.0 ± 2.4 (89%)	1.9 ± 0.1 (49%)	1.1 ± 0.1 (52%)
COL::Newman *prs*	121.7 ± 3.0 (78%)	40.7 ± 1.6 (98%)	2.5 ± 0.1 (64%)	1.2 ± 0.1 (57%)
COL::Newman *gltX*	129.8 ± 5.4 (83%)	44.2 ± 0.7 (106%)	2.8 ± 0.4 (72%)	1.0 ± 0.1 (48%)
COL *rpoB*$^+$	159.6 ± 3.6 (102%)	48.7 ± 1.4 (117%)	3.7 ± 0.1 (95%)	2.9 ± 0.1 (138%)
COL::Newman *prs*::Newman *gltX*	120.8 ± 3.8 (78%)	42.1 ± 1.3 (101%)	2.0 ± 0.5 (51%)	1.7 ± 0.1 (81%)
COL *rpoB*$^+$::Newman *prs*	125.7 ± 3.6 (80.7%)	42.2 ± 2.0 (101%)	1.3 ± 0.1 (33%)	0.9 ± 0.4 (43%)
COL *rpoB*$^+$::Newman *gltX*	127.6 ± 2.0 (82%)	40.0 ± 2.9 (96%)	1.6 ± 0.1 (41%)	1.7 ± 0.2 (81%)
COL *rpoB*$^+$::Newman *prs*::Newman *gltX*	110.3 ± 3.4 (71%)	35.8 ± 3.5 (86%)	2.4 ± 0.1 (62%)	1.4 ± 0.4 (67%)

[a]Percentages in parentheses indicate comparison with value for COL

Figures

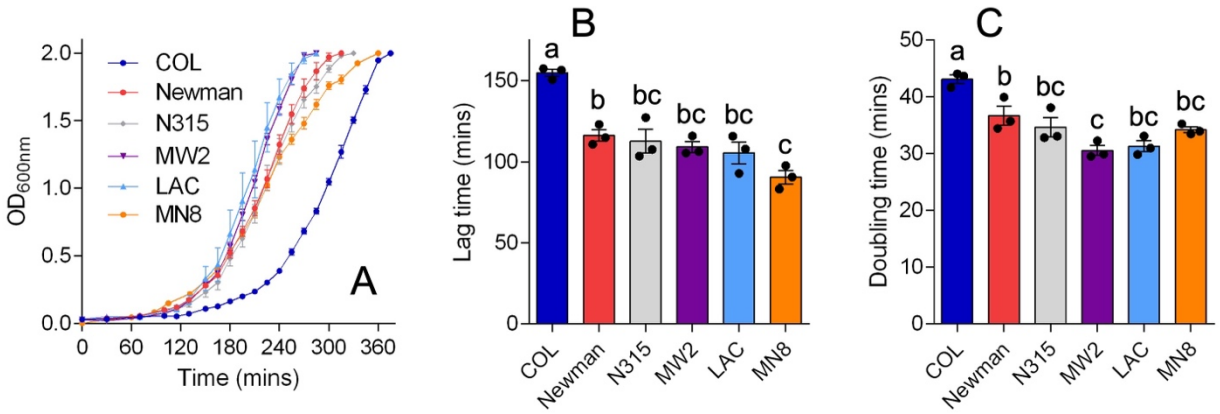

Figure 1. COL exhibits growth defects compared with other model strains of *S. aureus.* (A) Growth curves of six model *S. aureus* strains. Data shown are the mean of three biological replicates. Lag times (B) and doubling times (C) derived from the growth curves shown in panel A. All error bars, where visible, represent the SEM. Lowercase letters above bars indicate statically significant differences ($P<0.05$) between means as determined by a one-way ANOVA with Tukey's multiple comparisons test (*i.e.* bars bearing the same letter have means that are not significantly different from one another).

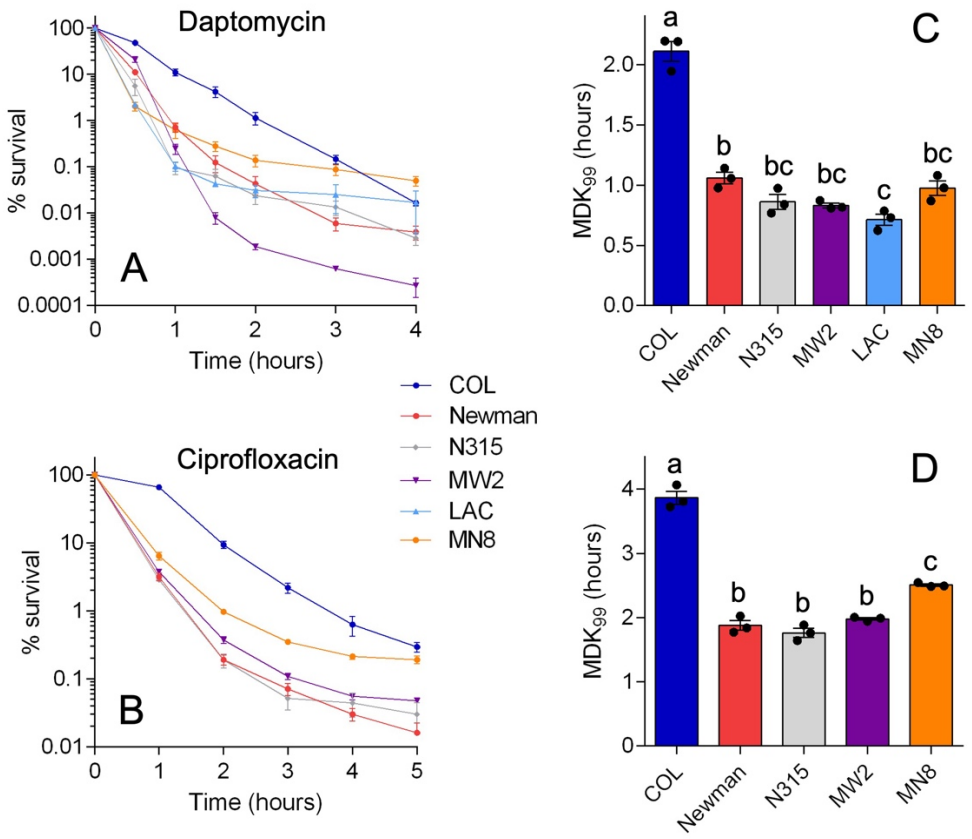

Figure 2. COL exhibits tolerance to daptomycin and ciprofloxacin compared with other model strains of *S. aureus.* Strains were exposed to (A) daptomycin or (B) ciprofloxacin and viable counts were determined at intervals. Counts at each time point are expressed as a percentage of the starting inoculum. Data shown are the mean of three biological replicates; error bars, where visible, represent the SEM. (C) and (D) Minimum duration for killing for 90% of the population (MDK_{99}) values were interpolated from the data shown in panels A and B. Lowercase letters above bars indicate statically significant differences ($P<0.05$) between means as determined by a one-way ANOVA with Tukey's multiple comparisons test (*i.e.* bars bearing the same letter have means that are not significantly different from one another). Strain LAC is resistant to ciprofloxacin (Table 1), so is not included in panels B and D.

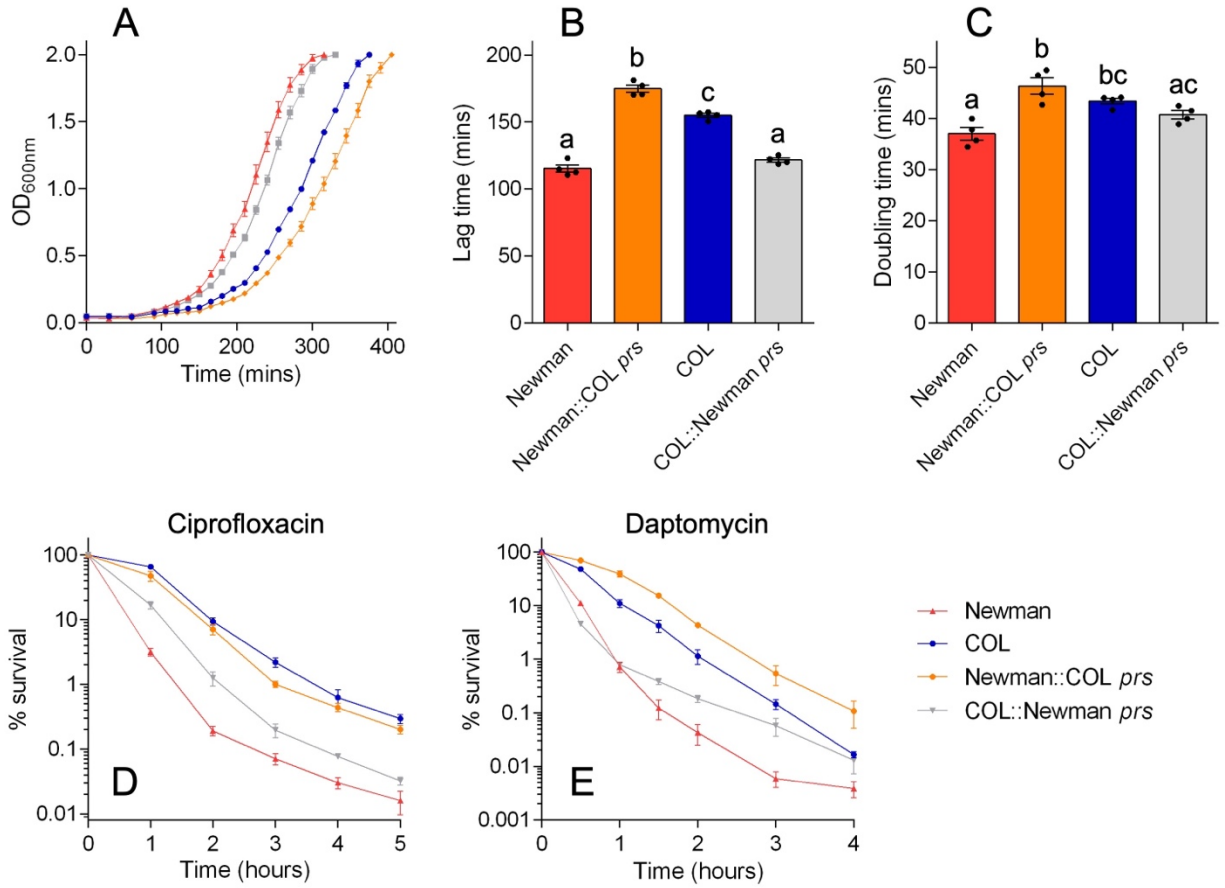

Figure 3. Impact of *prs* allele swapping on growth and tolerance. (A) Growth curves of wildtype COL, wildtype Newman and *prs* allele-swapped mutants. Lag times (B) and doubling times (C) derived from the growth curves shown in panel A. Lowercase letters above bars indicate statically significant differences ($P<0.05$) between means as determined by a one-way ANOVA with Tukey's multiple comparisons test (*i.e.* bars bearing the same letter have means that are not significantly different from one another). (D) and (E) Time-kills with 4 × MIC ciprofloxacin and daptomycin, respectively. Data shown are the mean of three (time-kill) or four (growth curves) biological replicates; error bars, where visible, represent the SEM. See Table 2 for MDK_{99} values derived from data shown in panels D and E.

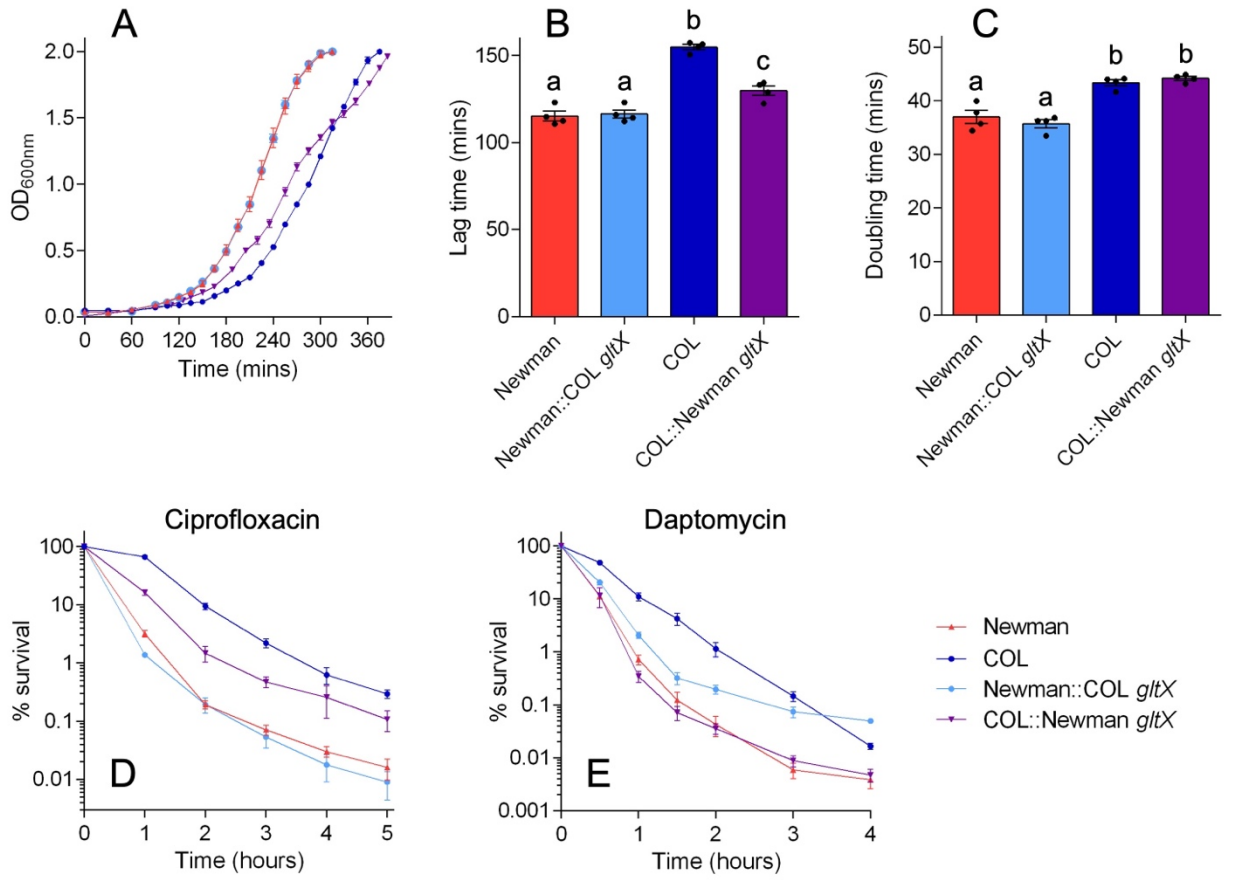

Figure 4. Impact of *gltX* allele swapping on growth and tolerance. (A) Growth curves of wildtype COL, wildtype Newman and *gltX* allele-swapped mutants. Lag times (B) and doubling times (C) derived from the growth curves shown in panel A. Lowercase letters above bars indicate statically significant differences ($P<0.05$) between means as determined by a one-way ANOVA with Tukey's multiple comparisons test (*i.e.* bars bearing the same letter have means that are not significantly different from one another). (D) and (E) Time-kills with ciprofloxacin and daptomycin, respectively. Data shown are the mean of three (time-kill) or four (growth curves) biological replicates; error bars, where visible, represent the SEM. See Table 2 for MDK_{99} values derived from data shown in panels D and E.

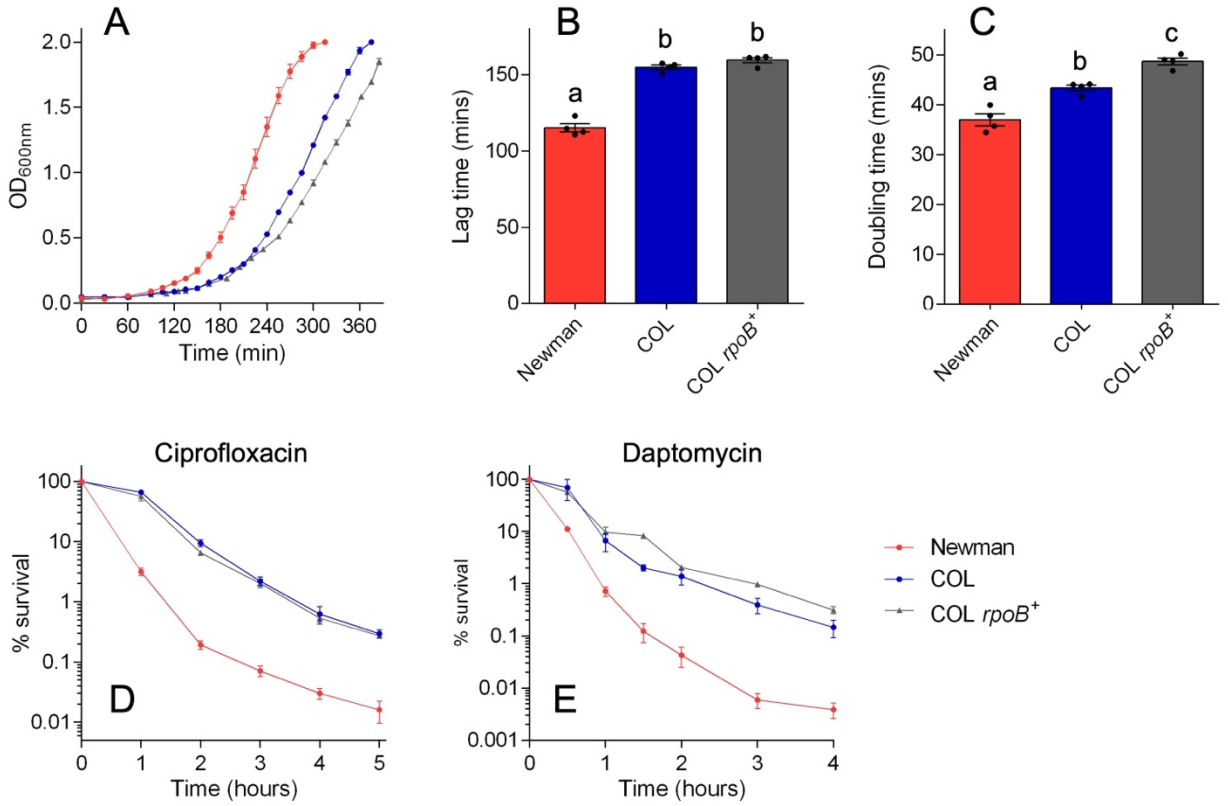

Figure 5. Impact of *rpoB* mutations on the growth and tolerance of COL. (A) Growth curves of wildtype COL, wildtype Newman and COL *rpoB*$^+$ (a variant of COL that carries the Newman *rpoB* allele, as well as a kanamycin resistance gene) grown in TSB. Lag times (B) and doubling times (C) derived from the growth curves shown in panel A. Lowercase letters above bars indicate statically significant differences (P<0.05) between means as determined by a one-way ANOVA with Tukey's multiple comparisons test (*i.e.* bars bearing the same letter have means that are not significantly different from one another). (D) and (E) Time-kills with ciprofloxacin and daptomycin, respectively. Data shown are the mean of three (time-kill) or four (growth curves) biological replicates; error bars, where visible, represent the SEM. See Table 2 for MDK_{99} values derived from data shown in panels D and E.

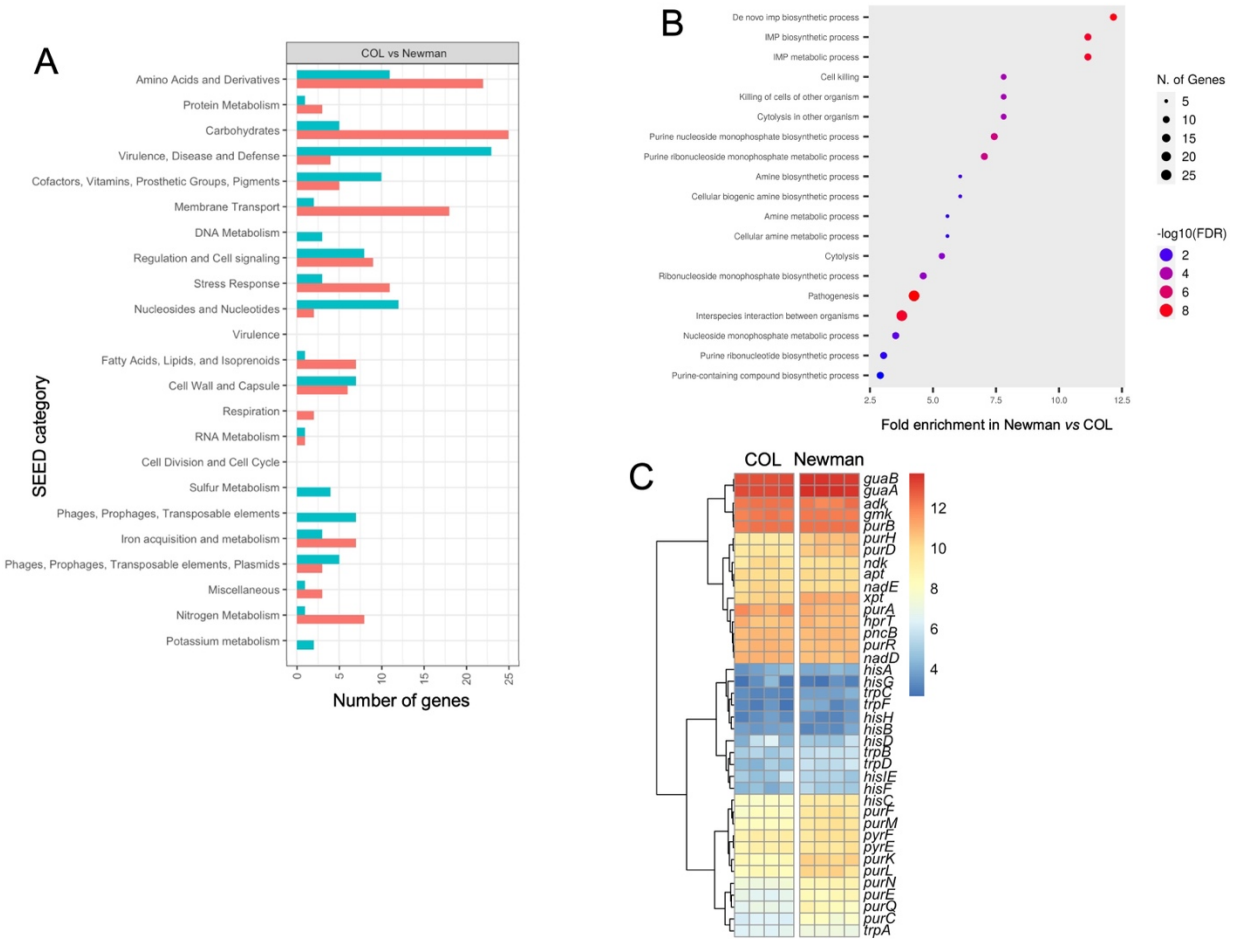

Figure 6. Transcriptomic profile of COL compared with Newman. (A) Functional classes of genes up- (red) and down-regulated (blue) in COL compared with Newman. (B) Gene ontology (GO) enrichment analysis showing that inosine monophosphate (IMP) and purine metabolism account for many of the GO terms that are over-represented in Newman compared with COL. (C) Heatmap comparing the expression of genes downstream of *prs* in COL and Newman. Counts are shown on a $\log_2$ scale. Transcriptomic data are derived from four biological replicates per strain. See Figure S5 for a schematic diagram of the metabolic pathways downstream of Prs. Abbreviation: inosine monophosphate (IMP).