A genome-wide atlas of antibiotic susceptibility targets and pathways to tolerance

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Detailed knowledge on how bacteria evade antibiotics and eventually develop resistance could open avenues for novel therapeutics and diagnostics. It is thereby key to develop a comprehensive genome-wide understanding of how bacteria process antibiotic stress, and how modulation of the involved processes affects their ability to overcome said stress. Here we undertake a comprehensive genetic analysis of how the human pathogen *Streptococcus pneumoniae* responds to 20 antibiotics. We build a genome-wide atlas of drug susceptibility determinants and generated a genetic interaction network that connects cellular processes and genes of unknown function, which we show can be used as therapeutic targets. Pathway analysis reveals a genome-wide atlas of cellular processes that can make a bacterium less susceptible, and often tolerant, in an antibiotic specific manner. Importantly, modulation of these processes confers fitness benefits during active infections under antibiotic selection. Moreover, screening of sequenced clinical isolates demonstrates that mutations in genes that decrease antibiotic sensitivity and increase tolerance readily evolve and are frequently associated with resistant strains, indicating such mutations could be harbingers for the emergence of antibiotic resistance.
The emergence of antibiotic resistance in bacterial pathogens is a continuously developing complex problem that is only solvable if besides new drugs we also learn to understand the exact (genetic) processes that enable resistance. For instance, new antibiotics and treatment strategies are key to retain the ability to treat resistant infections. However, a comprehensive understanding of how and under which conditions resistance emerges, which genes and pathways contribute to drug sensitivity, and how resistance may be prevented or even taken advantage of, are equally important, as it could make treatments more focused and possibly less dependent on new drugs. For many antibiotics, we know which genomic changes can cause resistance. However, it is often not clear how we get there with respect to which evolutionary paths are taken and whether for instance tolerance or lowered drug sensitivity precedes resistance. Interestingly, clinical strains isolated during antibiotic treatment failure may lack known resistance markers and instead contain multiple or lowered drug sensitivity precedes resistance. However, whether these changes play a role or not is often unclear because the distribution of changes that can affect a bacterium’s drug sensitivity are largely unknown. Therefore, understanding which genes, pathways and processes can contribute to altered drug susceptibility, could help identify genomic changes that not only sensitize bacteria to certain drugs, but desensitize them and may thereby act as precursors for antibiotic escape and/or resistance development.

Resistance emerges primarily through drug target mutations blocking antibiotic lethal action, upregulation of efflux pumps, and the acquisition of drug inactivating enzymes. Importantly, an antibiotic’s effects go far beyond the interaction with its direct target. We, and others, have shown that when a bacterium is challenged by an antibiotic, the imposing stress can expand throughout the bacterium and affect and demand the involvement of many different processes. For instance, while fluoroquinolones like ciprofloxacin inhibit DNA replication by targeting gyrase and/or topoisomerase, this also triggers double-stranded breaks requiring the involvement of DNA repair mechanisms, which in turn requires nucleotide and energy metabolism. Antibiotics can thereby trigger a stress cascade, that with mounting stress increasingly reverberates through the organismal network, until the accumulating stress passes a threshold at which point the organism succumbs to the pressure. This explains why mutations in genes or pathways involved in dealing with the downstream (indirect) effects of antibiotic exposure can often make a bacterium more sensitive to a specific antibiotic. Indeed, we have shown for Streptococcus pneumoniae and Acinetobacter baumannii that, for instance, targeting DNA repair makes bacteria more susceptible to DNA synthesis inhibitors (DSIs), or targeting the Rod-system and/or Divisome makes A. baumannii more sensitive to cell-wall synthesis inhibitors (CWSIs). This means that downstream pathways, genes and processes can be used as new targets or drug potentiators, either by themselves or in combination with others. Moreover, in most bacteria, as in any other organism, the majority of genes are of unknown function, it is unclear what role they play in a specific process and/or pathway, or how they are connected within the organismal genomic network. Thus, besides solving gene function, mapping-out which genes, pathways and processes are involved in dealing with and overcoming antibiotic stress, and how they interact with each other, can provide key insights into uncovering new drug targets, or for instance rational combination strategies.

While identifying off-target genes and pathways that increase drug sensitivity may thus be useful, it is possible that changes in associated processes could, in contrast, just as well reduce the experienced antibiotic stress. Such changes would thereby decrease antibiotic sensitivity and could possibly function as precursors to the emergence of resistance. A possible example of this is tolerance and/or persistence, where a small proportion of cells in a population can be induced by external conditions including nutrient starvation, cell density, antibiotic stress, and stress from the immune system into a cell state that enables them to tolerate high (transient) concentrations of antibiotics. Cell states associated with tolerance include cell dormancy, slow growth, transient expression of efflux pumps, and induction of stress response pathways. However, the mechanistic underpinnings of tolerance and decreased antibiotic sensitivity remain largely undefined and possibly differ between bacterial species and vary among antibiotics. Moreover, specific mutations can (dramatically) increase the fraction of the surviving population, indicating these tolerant phenotypes have a genetic basis. Lastly, since clinical isolates often carry mutations located outside well-characterized drug targets, they could thus be composed of variants with different antibiotic sensitivities. Consequently, such variants with decreased antibiotic sensitivity could enable antibiotic escape, and/or enable multi-step high-level resistance mutations to evolve as they are given an extended opportunity to emerge. Variants with decreased antibiotic sensitivity may thereby play an important role in antibiotic treatment failure. However, the breadth of possible genetic alterations that can enable (increased) tolerance and/or decrease antibiotic sensitivity are largely unknown, making it unclear how often and probable it is that such variants arise.

In this study, we use Tn-Seq in S. pneumoniae exposed to 20 antibiotics, 17 additional environments, and two in vivo infection conditions, to generate a genome-wide atlas of drug susceptibility determinants and build a genome-wide interaction network that connects cellular processes and genes of unknown function. We explore several interactions as new leads for gene function, while we show that specific interactions can be used to guide the identification of targets for new antimicrobial strategies. We highlight one such novel target in the membrane, by successfully developing a combinatorial antibiotic-antibody strategy that significantly reduces the bacterial load during an acute mouse lung infection. Furthermore, detailed mapping of antibiotic sensitivity data to pathways and genes with known function suggests a multitude of genome-wide genomic changes exist that can make the bacterium less susceptible and often tolerant to specific antibiotics. We untangle some of the underlying genetic mechanisms and show that decreased susceptibility and tolerance can come from a variety of changes including those in (nucleotide) metabolism, (p)ppGpp and ATP synthesis, transcription, and translation, as well as different types of transport. By further combining in vivo-infection- with antibiotic-Tn-Seq data we predict and experimentally validate that many disruptions may retain their decreased antibiotic sensitivity phenotype in vivo, and thereby outcompete the wild type in the presence of antibiotics. Moreover, by screening hundreds of clinical isolates we show that changes in genes that can decrease antibiotic sensitivity readily evolve in human patients and are often associated with antibiotic resistance. Consequently, these data highlight the wide array of possibilities that can lead to lowered antibiotic sensitivity and/or tolerance and underscore the importance of understanding the genetics of variants with altered drug susceptibility.

Results
A genome-wide view of antibiotic sensitivity. To obtain a genome-wide view of the genetic determinants that can modulate antibiotic stress in S. pneumoniae, Tn-Seq was employed in the presence of 20 antibiotics (ABXs), representing 9 different ABX groups and four classes including cell wall synthesis inhibitors.
(CWSIs), DNA synthesis inhibitors (DSIs), 30S and 50S protein synthesis inhibitors (PSIs) and an RNA synthesis inhibitor (RSI) (Fig. 1a). Six independent transposon libraries were generated and grown for approximately 8 generations in the absence and presence of an antibiotic at a concentration that reduces growth by approximately 30–50% (Supplementary Data 1). Tn-mutant frequencies are determined through Illumina sequencing from the beginning and end of the experiment with high reproducibility between libraries ($R^2 = 0.70–0.90$; Supplementary Fig. 1) which is consistent with previous Tn-Seq experiments. Combined with the population expansion during the experiment each mutant’s fitness ($W_{MT}$) is calculated to represent their environment-specific relative growth rate, which means that a mutant with for instance a fitness of 0.5 ($W_{MT} = 0.5$) grows twice as slow as the wild type (WT). Each gene’s antibiotic-specific fitness is statistically compared to baseline fitness without ABXs, and is represented as $\Delta W = W_{ABX} - W_{noABX}$ and categorized as: (1) Neutral, $\Delta W = 0$, a mutant’s relative growth is similar in the absence and presence of an ABX; (2) Negative, $\Delta W < 0$, a mutant’s fitness is significantly lower and thus grows relatively slower in the presence of an ABX; (3) Positive, $\Delta W > 0$, a mutant’s fitness is significantly higher and thus grows relatively faster in the presence of an ABX. All antibiotics trigger both positive and negative fitness effects (Fig. 1b, Supplementary Data 2), which are distributed across 22 different gene categories (Fig. 1c). Importantly, enrichment analysis shows there are multiple expected patterns, for instance genes involved in DNA repair are enriched in the presence of fluoroquinolones; cell-wall, peptidoglycan, and so on. Enrichment analysis shows that some pathways/processes such as glycolysis are relatively often involved in modulating responses to antibiotics, while other processes are more specific. Validated growth experiments performed throughout the project highlight the Tn-Seq data is of high quality. ±SEM are shown for each data point. Source data are provided as a Source Data file.
cell-division genes are enriched in β-lactams and glycopeptides; membrane integrity genes in lipopeptides; and transcription and translation in PSIs (Fig. 1d). Additionally, throughout the manuscript, we validate a total of 49 predicted genotype × phenotype interactions, which indicates the Tn-Seq data is of high quality and in line with previously shown accuracy

**Co-fitness interaction networks identify known and unknown genetic relationships.** Screens such as Tn-Seq are geared toward highlighting the genetic regions and/or genes that are important under a specific screening condition. With increasing conditions, genes acquire profiles that reflect their involvement/importance in those conditions, where genes with similar profiles indicate having similar and/or shared tasks. Such profiles can thereby help fill gaps in pathways, and/or identify genes and gene clusters with similar roles. By building a correlation matrix based on each gene’s ABX fitness-profile patterns emerge along a similarity range; from genes with highly similar to contrasting profiles. Moreover, to increase statistical power (i.e., more conditions increases the ability to identify more and stronger associations) the ABX dataset was supplemented with previously collected Tn-Seq data from 17 additional non-antibiotic conditions

**Detailed pathway mapping identifies processes that simultaneously increase and decrease drug susceptibility in an antibiotic-specific manner.** Two hundred and twenty-four genes with a known annotation are present in the data that have at least one significant phenotype in response to an antibiotic, which can be split over 21 functional groups according to a pathway or process they belong to (Fig. 3a). Each group is characterized by having multiple instances of decreased fitness, indicating genes that upon disruption increase sensitivity to one or more antibiotics (negative phenotype). Additionally, each group, except for cell division, also has multiple instances that increase fitness, which is suggestive of genes that upon disruption decrease antibiotic sensitivity (Fig. 3a; positive phenotype). Moreover, each antibiotic group triggers both negative and positive effects (Fig. 3b). Where possible, the 21 functional groups are organized according to a pathway or process they belong to and each gene is combined with its antibiotic susceptibility profile. This results in an antibiotic susceptibility atlas, which shows on a fine-grained scale, how inhibiting a pathway or process can seemingly simultaneously lead to increased and decreased drug susceptibility in an antibiotic-specific manner (Fig. 3c and Supplementary Figs. 2 and 3). For instance, in the glycolysis group, knocking out any of the three genes involved in forming the phosphotransferase (PTS)-system (SP_0282-SP_0284) that imports glucose to generate glucose-6-phosphate (G-6P), has a negative effect on fitness in the presence of 30S and 50S PSIs as well as Synercid (a synergistic combination of two PSIs), while it increases fitness in the presence of all CWSIs (β-lactams, glycopeptides, and daptomycin) and fluoroquinolones. Also, knocking out SP_0668 (gki; glucokinase), an enzyme that converts α-D-Glucose into G-6P, has a positive effect on fitness in all CWSIs and a negative effect in 30S PSIs. In contrast, inhibiting SP_1498 (pgm, phosphoglucomutase), the major interconversion enzyme of G-6P and G-1P, has a negative effect on fitness with all antibiotics (Fig. 3c).

Additional detailed examples are highlighted in Fig. 3c, for instance for pyruvate metabolism, where inhibiting lactate, or acetaldehyde production increases sensitivity to β-lactams and glycopeptides and decreases sensitivity to 30S PSIs, inhibiting formate production decreases sensitivity to co-trimoxazole and 30S PSIs, and inhibiting acetyl-phosphate production decreases sensitivity to β-lactams, glycopeptides, and co-trimoxazole. Within aspartate metabolism a range of changes can be triggered from increased sensitivity to β-lactams, and glycopeptides, to decreased sensitivity to most other antibiotics. Moreover, the four genes involved in the production of threonine from L-aspartate trigger decreased sensitivity to fluoroquinolones and 30S and 50S PSIs. In the shikimate pathway inhibiting the production of chorismate from phosphoenolpyruvate (PEP) and erythrose-5-phosphate leads to increased sensitivity to β-lactams, co-trimoxazole, and Synercid. Cell division is the only process that upon interference, only generates increased sensitivity, specifically to CWSIs and co-trimoxazole. Interfering with peptidoglycan synthesis also mostly leads to increased sensitivity to CWSIs, as well as to 30 S PSIs, while changes to genes that are involved in anchoring proteins to the cell wall (SP_1218 [srtA], SP_1833) can decrease sensitivity to CWSIs. Lastly, interfering with proteolytic activity, for instance through the protease complex ClpCP (SP_2194, SP_0746) and the regulator CtsR (SP_2195), is generally assumed to be fundamental for responding to stress,

**cozEb** encodes a cell division and peptidoglycan synthesis embedded membrane protein that can be critically targeted in vivo through an antibiotic-antibiotic strategy. By identifying
targets that (re)sensitize bacteria against existing antibiotics, genome-wide antibiotic susceptibility data have the potential to guide the development of new antimicrobial strategies. One such strategy could be a combined therapeutic antibody-antibiotic approach; the antibody would target a gene product that is important for sensitivity to one or more antibiotics and ideally the product would be easily accessible for the antibody at the bacterial cell surface. To find suitable candidate targets, Tn-Seq data were filtered for gene products that, based on a known function or localization prediction, are likely to be present in the cell wall or membrane, and that when disrupted, increase sensitivity to one or more antibiotics. Moreover, it would likely be ideal if the gene is also important for survival in vivo. A strong candidate is SP_1505, which in the interaction network is most tightly linked to cell wall metabolism and cell-division genes (Fig. 4a). After we previously hypothesized that it may play a role in cell wall integrity, it was recently named cozEb, with a likely role in organizing peptidoglycan synthesis during cell division, which fits its interaction profile (Fig. 4a). Importantly, the antibiotic Tn-Seq data suggest that disruption creates increased sensitivity to vancomycin and rifampicin, while the product is critical in the presence of daptomycin, which was confirmed through individual growth curves (Fig. 4b). The protein has eight predicted membrane-spanning domains (Fig. 4c), and in vivo Tn-Seq predicts it is important for survival in both the nasopharynx and lungs (Fig. 4a, Supplementary Data 2). The gene was cloned into an expression plasmid generating an ~30kD product (Fig. 4c), which was used to raise rabbit anti-CozEb antibodies, which were confirmed to be specific for the cozEb gene product (Fig. 4c). Potential antibody in vitro activity was determined through a bacterial survival assay in the absence and presence of antibodies and either vancomycin or daptomycin. Incubating bacteria with antibodies or daptomycin has no significant effect on bacterial survival, while vancomycin alone at significantly lower concentration used slightly reduces the number of surviving bacteria. Moreover, combining the antibody with either vancomycin or daptomycin further reduces the number of surviving bacteria in vitro compared to any agent individually (Fig. 4d). To assess whether the antibody-antibiotic approach works in vivo, mice were intranasally challenged with a bacterial inoculum either containing WT or ΔcozEb. Two additional sets of mice were challenged with WT and 8 h post infection they were either treated with daptomycin and control IgG antibody or with daptomycin and CozEb-specific antibody. Mice were sacrificed 24 h post infection, and bacteria in the lungs were enumerated. As predicted by the in vivo Tn-Seq data the cozEb knockout has a significantly lower fitness in the lungs highlighted by an up to
2.5-log lower bacterial load compared to WT. Importantly, while the WT survives equally well in the presence of the low daptomycin concentration and the control IgG antibody, in the presence of daptomycin and the CozEb-targeting antibody, its survival in the lungs is significantly reduced and resembles that of the cozEb knockout (Fig. 4e). This shows that by combining antibiotic and in vivo Tn-Seq with gene annotation information, a gene product can be selected that is central and critical to cell-wall synthesis and cell-division processes. Importantly, due to its presence in the membrane, it is directly targetable with an antibiotic, thereby sensitizing the bacterium to an antibiotic concentration that is normally not sensitive to it.

The ami-operon encodes an antibiotic importer, and inhibition triggers tolerance. The example above illustrates how negative fitness indicates increased antibiotic sensitivity reflected by reduced relative growth, which can guide the development of (re)sensitizing approaches. In contrast, the occurrences of increased fitness in the dataset indicate that a large number of options exist that could lead to reduced antibiotic sensitivity (Fig. 3). With increased fitness to 3 out of 4 antibiotic classes, the ami-operon is among genes with the greatest number of positive fitness effects. The operon forms a tight cluster in the interaction network (Figs. 3 and 5a) and it is annotated as an oligopeptide transporter with no clear function. Two separate knockouts for SP_1888 (amiE) and SP_1890 (amiC) confirm that increased fitness results in decreased drug sensitivity in the form of increased relative growth in the presence of ciprofloxacin, vancomycin and gentamicin, and increased sensitivity (i.e., decreased relative growth) to Synercid (Fig. 5b). There is limited evidence that the ami-transporter may have (some) affinity for at least two different peptides (P1 and P2). These have been theorized to possibly function as signaling molecules and

| Antibiotic Group/Class       | Positive | Negative |
|------------------------------|----------|----------|
| 1. B-lactams                 |          |          |
| 2. Glycopeptides             |          |          |
| 3. Lipoprotein               |          |          |
| 4. Fluoroquinolones          |          |          |
| 5. Cotrimoxazole             |          |          |
| 6. 30S PSIs                  |          |          |
| 7. 50S PSIs                  |          |          |
| 8. Synercid                  |          |          |
| 9. Rifampicin                |          |          |
| 10. Glucose biosynthesis     |          |          |
| 11. Cell division            |          |          |
| 12. Peptidoglycan, biosynthesis |        |          |
| 13. Wall/Lipoteic acid biosynthesis | | |
| 14. Membrane integrity       |          |          |
| 15. DNA Repair               |          |          |
| 16. Transcription & Translation |      |          |
| 17. Protein turnover         |          |          |
| 18. C-di-AMP synthsis        |          |          |
| 19. Regulation              |          |          |
| 20. Calcium/Metal Transport  |          |          |
| 21. Various Transport        |          |          |

| Process / Group | Genes | Negative | Positive |
|-----------------|-------|----------|----------|
| 1. Glycolysis    |        |          |          |
| 2. Pyruvate metabolism | | |
| 3. Pentose Phosphate Pathway | | |
| 4. Aspartate metabolism | | |
| 5. Shikimate Pathway | | |
| 6. Ascorbate metabolism | | |
| 7. Folate biosynthesis | | |
| 8. Purine metabolism | | |
| 9. Capsule biosynthesis | | |
| 10. Glucose biosynthesis | | |
| 11. Cell division | | |
| 12. Peptidoglycan, biosynthesis | | |
| 13. Wall/Lipoteic acid biosynthesis | | |
| 14. Membrane integrity | | |
| 15. DNA Repair | | |
| 16. Transcription & Translation | | |
| 17. Protein turnover | | |
| 18. C-di-AMP synthsis | | |
| 19. Regulation | | |
| 20. Calcium/Metal Transport | | |
| 21. Various Transport | | |

**Fig. 3** A multitude of options, pathways, and processes can simultaneously lead to increased and decreased antibiotic susceptibility. a. The number of phenotypes scored for each pathway/process. Genes with at least one significant phenotype are split over 21 groups according to a pathway or process they belong to, which highlights how modulation of most pathways can lead to increased (negative phenotype) and decreased (positive phenotype) antibiotic sensitivity. b. The number of phenotypes scored for each antibiotic group. While sensitivity to each antibiotic (group) can be increased by knocking out genes in the genome (negative phenotype), sensitivity can be decreased (positive phenotype) almost as often for most ABXs, except for vancomycin and gentamicin, and increased sensitivity (i.e., decreased relative growth) to Synercid. c. Detailed view of 7 out of 21 groups/processes highlighting how modulation of specific targets within each group leads to changes in antibiotic sensitivity. Each group is indicated with a number that is the same as in a. Where possible, genes are ordered according to their place in a process/pathway, and gene numbers (SP_) are combined with gene names and annotation. Each indicated gene is combined with an ‘antibiotic sensitivity bar’ indicating whether disruption leads to increased (red/negative phenotype) or decreased (green/positive phenotype) sensitivity to a specific group of antibiotics. When phenotypic responses are the same, multiple genes are indicated with a single bar (e.g. SP0028/SP0283/SP0284 in glycolysis, or SP0413/SP1013/SP1361/SP1360 in Aspartate metabolism). Gene numbers in blue have no effect on growth in the absence of antibiotics, but knockout results in a significant decrease in the absence of ABXs (see for detailed fitness in the absence and presence of antibiotics Supplementary Data 2). Essential genes are not indicated and genes with an asterisk have a partial or tentative annotation that has not been resolved. All 21 groups are listed in Supplementary Figs 2 and 3. Source data are provided as a Source Data file.
under certain circumstances may be generated by the bacterium itself51–53. Both peptides were synthesized and while neither peptide affects growth of the WT or knockout mutants in the absence of antibiotics (Supplementary Fig. 4), the WT grows slightly better in the presence of gentamicin and peptide P2, but not P1 (Fig. 5b). This shows that some peptides may, at least partially, inhibit or occupy the ami-transporter, and thereby trigger decreased antibiotic sensitivity, in a similar manner as a knockout does. Besides peptides, the ami-transporter may be (non-selectively) transporting antibiotics into the cell, which could explain its effect on antibiotic sensitivity. To explore this, bacteria were exposed to ciprofloxacin or kanamycin and the internalized antibiotic concentration was determined through mass spectrometry for WT and both ami knockout mutants. In both mutants, the amount of internalized ciprofloxacin or kanamycin was significantly lower (~1.7× in ΔamiE, and ~2.3× in ΔamiC), while the kanamycin concentration was found to be significantly lower in ΔamiC (~2×; Fig. 5c). This shows that a functional ami-transporter increases the concentration of fluoroquinolones and 30S PSIs, suggestively by transporting them into the cell, and thereby, due to a higher internal concentration, enhancing the antibiotic’s inhibitory effects on growth. There are multiple examples that transporters can contribute to tolerance54,55, which we recently showed is also the case for the ade transporter in Acinetobacter baumannii, which contributes to fluoroquinolone tolerance7. However, those examples are mostly based on efflux pumps that actively decrease the antibiotic concentration in the cell through upregulation of such pumps. In contrast, with respect to the ami-operon it would be the reverse, i.e., inhibition instead of upregulation would lead to tolerance. To explore this possible effect on tolerance, the WT and ΔamiE were exposed to either 10xMIC of gentamicin or vancomycin over a period of 24 h. Approximately 1% of the WT population survives 4 h exposure to gentamicin, while none of the population survives exposure past 8 h. The ΔamiE population displays a slower decline in survival with 1% of the population surviving past 8 h (tolerant cells)25. At ~10 h the decline ceases and the remaining population (~0.01%) survives at least up to 24 h, which is representative of a persister fraction25. In contrast, the WT and amiE mutant populations decline at similar rates when exposed to vancomycin, showing that inhibition of the ami-transporter can lead to tolerance and persistence in an antibiotic-specific manner while MICs of gentamicin and vancomycin for WT and ΔamiE are similar (Supplementary Data 1). Importantly, these data show that increased fitness indeed leads to decreased ABX sensitivity, which can translate into at least two phenotypes: increased relative growth and increased survival (i.e., tolerance).

Fig. 4 CozEb an integral membrane protein increases antibiotic sensitivity and can be targeted with an antibody. a cozEb/SP_1505 is tightly clustered with cell division and cell wall metabolism genes, it is predicted to increase sensitivity to glycopeptidase and the lipopeptide daptomycin, and has a decreased fitness in the mouse lung and nasopharynx. b Reduced relative growth of ΔcozEb validates its increased sensitivity to daptomycin and vancomycin. c CozEb has 8 transmembrane domains, which generates a ~30 Kd product (BSA is shown as a control). The cloned protein was used to raise antibodies, which proved to be specific for a product in the WT membrane, but does not bind anything in ΔcozEb, indicating the antibodies are specific for the membrane protein CozEb. d Incubation of WT for 2 h with vancomycin (Vanco) or daptomycin (Dapto) and in the presence of CozEb antibody, slightly decreased antibiotic sensitivity, in a similar manner as a knockout. e An in vivo lung infection with WT or ΔcozEb confirms the mutant is less fit in vivo. Challenging the WT with daptomycin and IgG does not affect bacterial survival. In contrast, challenging with daptomycin and CozEb-specific antibodies, significantly reduces the recovered CFUs 24 h post infection. Mean values ± SEM are shown from n ≥ 10 mice/experiment. Significance is measured through a one-way ANOVA with Dunnett correction for multiple testing: *p = 0.03, **p = 0.001, ***p < 0.001. Source data are provided as a Source Data file.
Purine metabolism, (p)ppGpp and ATP production are tightly linked to altered ABX susceptibility and tolerance. Among the 21 functional groups, purine metabolism has some of the largest number of positive fitness effects, mostly with β-lactams and glycopeptides (Figs. 3a and 6a). Moreover, two regulators (SP_1821/1979) associated with this pathway decrease sensitivity to β-lactams and/or glycopeptides and two ‘neighboring’ genes with unknown function have either the same (SP_0830), or the opposite effect (SP_1446) on antibiotic sensitivity as their defined neighbor, suggesting they may be involved in the same process as their neighbor (Fig. 6a). Furthermore, the global interaction network positively links an ABC transporter (SP_0845-0848, Figs. 2c, 6a) with multiple genes in this pathway due to their similar profiles. This operon is annotated as a putative deoxyribose transporter, and to verify whether an interaction exists with purine metabolism, single and double knockouts were created between SP_0846 (the transporter’s ATP binding protein) and SP_0829/deoB. Their profiles suggest they do not affect growth in the absence of ABXs and have increased sensitivity to Synercid, which was confirmed with individual growth curves (Fig. 6b).

However, when both knockouts are in the same background, their increased sensitivity to Synercid is masked. Thus, as indicated by the network, these results show that the ABC transporter indeed has a genetic interaction with purine metabolism/salvage, but plays an unknown role. Importantly, this confirms that the global network includes valuable interactions that can be explored to uncover functional relationships.

Furthermore, within purine metabolism the alarmone (pp)Gpp is synthesized from GTP and/or GDP. Like other bacterial species, S. pneumoniae likely responds to (some) ABXs via induction of the stringent response pathway, in which relA (SP_1645) is the key player with both synthetase and hydrolase activity. Additionally, SP_1097 is annotated as a GTP diphosphokinase and may be involved in the synthesis of pppGpp from GTP (Fig. 6a). Our data suggests, and we confirmed for the β-lactam cefepime (Fig. 6c), that when synthesis of the alarmone is inhibited by deletion of relA, similar to many other interactions in purine metabolism, this leads to reduced β-lactam and glycopeptide sensitivity manifested by increased relative growth (Fig. 6c). Moreover, while SP_1097, as predicted, does not...
change ABX sensitivity (Supplementary Data 2, Fig. 6), a double knockout of relA-SP_1097 seems to further decrease sensitivity to cefepime by further increasing relative growth (Figs. 6c and 7a). Additionally, besides a change in growth, the single relA and double knockout (ΔrelA-SP_1097), also increases tolerance to cefepime by ~1000-fold at 24 h (Fig. 7b), without changing the MIC (Supplementary Data 1). To understand how relA and SP_1097 affect purine metabolism, we used LC/MS to measure (p)ppGpp, ADP, ATP, GDP, and GTP. Additionally, we included SP_0831 a purine nucleoside phosphorylase involved in nucleotide salvage, which has the same ABX profile as ΔrelA (Fig. 6a, d), but should not directly affect (p)ppGpp synthesis. While (p)ppGpp is below the limit of detection during normal growth in any of the strains, as expected ΔrelA and the double mutant ΔrelA-SP_1097 are unable to synthesize the alarmone when exposed to mupirocin, a strong activator of the stringent response (Fig. 6e, Supplementary Data 9). In contrast, WT, ΔSP_0831, and ΔSP_1097 synthesize (p)ppGpp upon mupirocin exposure to a similar extent (Fig. 6e). Concerning the di- and trinucleotides in the pathway, upon mupirocin exposure GTP and GDP are significantly reduced in WT, ΔSP_0831, and ΔSP_1097, likely because they are used for (p)ppGpp synthesis (Fig. 6f, Supplementary Data 9). In contrast, while ATP and ADP again remain constant for the ΔrelA mutants, ATP and ADP synthesis are significantly increased upon mupirocin exposure, especially for WT and ΔSP_1097. This suggests that during activation of the
stringent response, synthesis from IMP is directed toward AMP, and not necessarily GMP, at least not enough to replenish GTP and GDP. Additionally, upon mupirocin exposure, ATP only minimally increases for ΔSP_0831, while it increases over twofold for WT and ΔSP_1097 (Fig. 6f). It has been shown for bacteria including Escherichia coli and Staphylococcus aureus that a decreased ATP concentration can decrease sensitivity to ABXs such as cipromoxacin58. Additionally, in S. aureus (p)ppGpp overexpression has been associated with decreased sensitivity to linezolid59. Our data suggest that (p)ppGpp and ATP synthesis may be intrinsically linked, i.e., at least in S. pneumoniae the inability to produce the alarmone also results in lowered ATP synthesis, which is associated with a lowered ABX sensitivity to β-lactams and glycopeptides. However, ΔSP_0831 shows that even if (p)ppGpp can be synthesized, modulation of purine metabolism, for instance through the salvage pathway, can result in decreased ATP synthesis, and can lead to lowered ABX sensitivity (i.e., increased relative growth). Importantly, in many bacterial species, alarmone production is generally assumed to be triggered in response to different types of stress and has been shown to affect a large variety of processes including nucleotide synthesis, lipid metabolism, and translation. (p)ppGpp is thereby a ubiquitous stress-signaling molecule that enables bacteria to generate a response that is geared toward overcoming the encountered stress. However, contradictory results between species indicate a possible non-uniformity across bacteria, leaving much to be learned about how the alarmone and the processes it can control fit into the entire organismal (response) network56.

There are a multitude of predictable pathways that lead to tolerance in vivo in an antibiotic dependent manner. For instance,
in the glycolysis. To further confirm that antibiotic sensitivity can be decreased by inhibiting a variety of processes, knockouts (KOs) were generated for fourteen mutants from 8 different processes. Moreover, an additional goal was to determine what increased fitness (i.e., decreased ABX sensitivity) would look like phenotypically, and thus whether it would translate into increased relative growth and/or tolerance. Of the 14 mutants with a Tn-Seq predicted increased fitness, 13 display an increased ability to grow in the presence of an ABX compared to the WT. Moreover, eight mutants, which inhibit several different processes including different metabolic pathways, transport, and transcription and translation, displayed tolerance, while retaining a similar MIC, and thereby have an increased ability to survive high-level exposure to an ABX (5–10xMIC) for at least 24 h (Fig. 7a, b, Supplementary Data 1,8). Note that we validated 49 single KO genotype × phenotype associations in this study, with an equal distribution across the entire spectrum of ABX sensitivity (Fig. 1e, Supplementary Data 8). This highlights that our approach uncovered a detailed genome-wide ABX sensitivity atlas composed of a mixture of genes, pathways and processes that when modulated can increase and/or decrease ABX sensitivity. The validation experiments highlight that the resulting fitness accurately predicts the relative growth rate of a mutant, which we have previously shown for hundreds of other negative fitness phenotypes,6,14–16,18,39–42,44,60–62 Moreover, it turns out that in the majority of cases, increased fitness not only results in increased relative growth in the presence of an antibiotic, but also tolerance. Thereby, the part of the atlas that depicts decreased ABX sensitivity (i.e., increased fitness) includes a genome-wide ‘tolerance’, composed of a wide variety of pathways and processes that when modulated trigger tolerance in vitro in an ABX dependent manner.

Obviously, the selection regime in vivo is far more complex and stricter than in a test tube, which raises the question whether many of the options that decrease ABX sensitivity in vitro, including those that increase tolerance, would be available in vivo as well. To explore this, all the Tn-Seq data with a positive fitness in the presence of at least one antibiotic was combined with in vivo Tn-Seq data and filtered for those genes with no or only a small fitness defect predicted in vivo during nasopharynx colonization or lung infection (Fig. 7c, Supplementary Data 2).

Two genes were selected that we had confirmed for decreased ABX sensitivity in vitro: (1) SP_0829/deoB synthesizes Ribose-1P and is involved in purine metabolism (Fig. 6a). ΔdeoB has no effect on in vitro growth in the absence of ABX (Fig. 7a, d), as predicted it grows better in the presence of cefepime (Fig. 7a, d), but it does not affect survival/tolerance (Fig. 7b); (2) SP_1396/pstA is the ATP binding protein of a phosphate ABC transporter (Supplementary Fig. 3). ΔpstA has no effect on in vitro growth (Fig. 7a, d), it has a higher relative growth rate in the presence of meropenem (Fig. 7a, d), and it also increases survival/tolerance (Fig. 7b). Both mutants were mixed with WT in a 1:1 ratio and used in an in vivo mouse infection competition model as we have done previously.18 Of the infected mice, half were administered antibiotics at 16 h post infection, and were sacrificed 6 h later to determine the strain’s competitive index (CI) (Fig. 7e). Importantly, while both mutants may have a slight disadvantage compared to the WT when colonizing the lung or nasopharynx, their CI increases significantly in the presence of ABXs, leading to increased survival compared to the WT (Fig. 7e, Supplementary Data 10). Combining antibiotic- with in vivo Tn-Seq highlights the ability to predict the existence of a wide array of possible alterations of specific genes, pathways and processes that can have a beneficial effect in vivo in the presence of antibiotics. Such changes could thereby contribute to escape from antibiotic pressure and even create a path toward the emergence of antibiotic resistance.

There is likely significant overlap in the selective pressures a bacterial pathogen would experience in a mouse infection model compared to the human host. This raises the possibility that those gene disruptions that are predicted by Tn-Seq to lead to decreased antibiotic sensitivity and that simultaneously have no more than a minimal defect in vivo, could also have an advantage in the human host in the presence of ABXs and thereby contribute to ABX escape and/or the emergence of resistance. A premature stop codon most closely reflects the effect a transposon insertion has on a gene; i.e., it disables a gene. We thereby hypothesized that stop codons in certain gene sets predicted by Tn-Seq could be enriched for in antibiotic-resistant clinical isolates. To test this hypothesis 4 gene sets were compiled consisting of those that upon disruption: (1) decrease antibiotic sensitivity in at least 1 ABX and have no strong defect in vivo; (2) decrease antibiotic sensitivity in at least 1 antibiotic and have a defect in vivo; (3) have little to no effect on antibiotic sensitivity and in vivo; (4) have no effect or increase antibiotic sensitivity and have a defect in vivo (Fig. 8a, b; Supplementary Fig. 5). Thousands of strains were selected from the PATRIC database that could be split into a group of co-trimoxazole (SXT) resistant and a group of β-lactam resistant strains, and each group was matched with an equal number of sensitive strains from the database. In all strains in the SXT and β-lactam groups, irrespective of resistant or sensitive status, the number of stop codons in gene sets 1 and 3 are highest, which reflects the Tn-Seq predicted in vivo effects, i.e., while gene sets 1 and 3 contain mostly genes with potentially neutral effects, gene sets 2 and 4 contain many genes that are suggested to have a defect in vivo when disabled (e.g. with a stop codon) (Fig. 8c). Moreover, SXT resistant isolates in gene set 1 more often contain a stop codon compared to sensitive strains, and in β-lactam resistant isolates this is true for gene sets 1–3 (Fig. 8d). While these are not ideal comparisons, for instance the entire ABX profile is not clear for many strains, different changes than premature stops could have ABX/in vivo modulating effects, strains could have experienced different ABX and/or in vivo selective pressures, and genetic changes can be strain-background dependent, it shows that genetic changes that can affect ABX and/or in vivo sensitivity, which are predictable with Tn-Seq, readily occur in clinical samples. This in turn underscores that ongoing infections may consist of variants that enable different paths to adjusting to, or overcoming a challenging host/ABX environment.

### Discussion

The emergence and increase in antibiotic resistance among most bacterial pathogens is a continuously developing problem with several important drivers, which include: (1) a lagging development of new drugs and treatment strategies; (2) a lack of (rapid) diagnostics and prognostics; and (3) an incomplete understanding of how antibiotic resistance develops. Moreover, these drivers are inherently connected making it a complex problem to solve. First, the ability of bacteria to evolve resistance elicits an arms-race that requires the development of new drugs and treatment strategies to keep the balance of infection-control tipped in our favor. Thus, while developing new drugs would keep the arms-race in place, the ability to slow or prevent the emergence of resistance could resolve the status quo. Furthermore, even though it is critical to understand how and under which circumstances resistance evolves, the applicability of this knowledge depends on the availability of diagnostics that could inform on the emergence of resistance (precursors) and thereby guide
and enable timely, tailored, and targeted treatments. To progress toward a comprehensive understanding of how an infection is developing in the absence or presence of treatment, and how to decide what to do next, we believe that a detailed genetic understanding of how a bacterium deals with and overcomes stress, as well as its genetic potential to achieve this, are key aspects. In this study, we contribute to reaching such an understanding by building and exploring a detailed atlas of ABX sensitivities, which highlights how modulation of specific genes, pathways and processes does not only result (as expected) in increased ABX sensitivity, but surprisingly often in decreased ABX sensitivity. We show that such an atlas can be used to identify leads for gene function, to uncover the genome’s underlying architecture and genetic relationships among genes, for the identification of new drug targets, and the development of new proof-of-principle antimicrobial (ABX sensitizing) strategies. Most importantly, these data identify genome-wide genetic changes that show how modulation of genes, pathways, and processes can lead to reduced antibiotic sensitivity (i.e., increased relative growth and tolerance), not only in vitro, but also in vivo. Moreover, we show that mutations that have the potential to trigger the same phenotypes readily occur in patients.

While the primary processes targeted by ABXs are mostly known, this work contributes to the increasing notion that downstream processes, not directly related to the target and which include metabolism, can significantly contribute to antibiotic efficacy. In E. coli it has been shown that inhibition of specific steps in purine metabolism can lead to decreased sensitivity to ampicillin and ciprofloxacin, and increased sensitivity to gentamycin. Our data explores a wider set of alterations in purine metabolism in S. pneumoniae, which also leads to a wider distribution of changes. However, the overlap with E. coli includes decreased sensitivity to β-lactams (an ABX class that includes ampicillin) and we show that inhibition of some reactions in purine metabolism can also lead to decreased sensitivity to fluoroquinolones (an ABX class that includes ciprofloxacin). Additionally, it has been shown for E. coli that antibiotic sensitivity to ampicillin and ciprofloxacin can be increased, at least over 4 h, by supplementing with adenine, but not guanine, which is possibly linked to a reduced ATP demand and synthesis. Our results show that limiting the ability to synthesize adenine, but also guanine, leads to significantly lowered ABX sensitivity to β-lactams and glycopeptides. Moreover, we show that limiting the ability to synthesize adenine, as well as

Fig. 8 Stop codons are enriched in clinical samples in Tn-Seq predicted tolerome genes. a Based on in vivo and ABX Tn-Seq data, four gene sets consisting of 34 genes each were compiled with specific fitness profiles in the presence of antibiotics and in vivo. Shown are the in vivo effects for nasopharynx, while lung data are depicted in Supplementary Fig. 5. ΔW represents the fitness difference of a gene in a specific condition (e.g., an antibiotic, in vivo) minus its fitness in vitro in rich medium. Dashed lines indicate significance cut-offs, grayed-out dots indicate genes with no significant change in fitness in the presence of antibiotics, colors represent antibiotics and are the same as in Fig. 1. b Detailed distributions for each gene set highlight whether effects in the presence of antibiotics, in the nasopharynx and lungs increase (+), do not affect (0) or decrease (−) relative fitness. Gene set rationales are described in the text. c The total number of stop codons in each gene set for 2296 co-trimoxazole and 1166 β-lactam resistant and sensitive strains. d The number of sensitive and resistant strains with at least one stop codon in a gene in each gene set. Significance is measured through a Fisher’s exact test: **p < 0.01, ***p < 0.001, ****p < 0.0001. Source data are provided as a Source Data file.
(p)ppGpp, leads to lowered ATP synthesis. The association between decreased ATP synthesis/availability and decreased sensitivity and/or tolerance to antibiotics including β-lactams, glycopeptides and fluoroquinolones has now been shown for a variety of Gram-positive and -negative organisms\textsuperscript{15,38,70,71}. While this suggests that low ATP demand/synthesis/availability may be at the root of a general mechanism that leads to decreased sensitivity to some antibiotics, our genome-wide atlas shows that decreased ABX sensitivity and tolerance to β-lactams, and glycopeptides can be triggered by alterations to pathways and processes other than those directly related to ATP synthesis, which include parts of glycolysis, pyruvate, ascorbate, glucose and purine metabolism, protein turnover and c-di-AMP synthesis (Supplementary Figs. 2 and 3). While, it is possible that many of these alterations do affect ATP demand, availability and/or synthesis, it is likely that they trigger a much more complex and varied set of changes. Importantly, the full extent of signals and (genetic) alterations that can lead to decreased ABX sensitivity, including tolerance, remain mechanistically poorly understood\textsuperscript{25,34}. This also means that it remains unclear whether there are common denominators or ‘universal rules’ that are applicable across strains and species\textsuperscript{25,34,35,58,70,71}. Different (computational) approaches are being explored to build such a comprehensive understanding\textsuperscript{6,15,17,69,72,73}. However, unequivocally more genome-wide data from more species remain needed. Thereby, the genome-wide insights we present here are helping build a rationale to measure and model this complexity. One goal of such models would be to obtain a detailed understanding and ability to predict how alterations to specific processes affect responses to ABXs and thereby drive changes in sensitivity\textsuperscript{15,17,25,34,69}. Lastly, these detailed data on reduced antibiotic sensitivities also suggest that more potential routes to ABX escape, and eventually resistance, may exist than assumed. We believe these data are thereby both an argument and potential starting point for a platform to predict clinically relevant mutations and determinants of antibiotic resistance and/or tolerance. Consequently, they underscore the importance of understanding the genetics of variants with altered drug susceptibility, as their genetics makes them diagnostically identifiable and trackable, while their often-associated collateral sensitivities to other ABXs or drugs could make them targetable.

### Methods

The research presented within complies with all relevant ethical regulations and protocols as approved by the Boston College environmental health and safety board.

#### Bacterial culturing, growth curves, and tolerance experiments

Experiments were performed with \textit{S. pneumoniae} strain TIGR4 (NCBI Reference Sequence: NC_003028.3). TIGR4 is a serotype 4 strain that was originally isolated from a patient from Norway with Invasive Pneumococcal Disease (IPD)\textsuperscript{32}. All S. pneumo gene numbers in the tables and figures are according to the TIGR4 genome. Single gene knockouts were constructed by replacing the coding sequence with a chloramphenicol and/or spectinomycin resistance cassette as described previously\textsuperscript{39,60}. \textit{S. pneumoniae} was grown on sheep’s blood agar plates or statically in THY, C + Y or semi-defined minimal media at pH 7.3, with 5 µL/mL Oxide (Oxide, Inc.) at 37 °C in a 5% CO\textsubscript{2} atmosphere\textsuperscript{35}. Where appropriate, cultures and blood plates contained 4 µg/mL chloramphenicol (Cm) and/or 200 µg/mL spectinomycin (Spec). Single strain growth assays were performed three times using 96-well plates by taking OD\textsubscript{600} measurements on a Tecan Infinite 200 PRO plate reader or BioSpa 8 (BioTek). Growth curves are fitted to an exponential growth equation to calculate doubling time. WT doubling time is divided by a mutant’s (MT) doubling time to represent each mutant’s fitness (i.e., relative growth rate; WT doubling time/MT doubling time = W\textsubscript{mutant}), making it directly comparable to Tn-Seq fitness\textsuperscript{40,48,63,46,60,62,67}. Tolerance experiments were performed by exposing exponentially growing bacteria to 5~100×MIC of an antibiotic at different time points (up to 24 h) and then washed with PBS, and plated on blood agar for enumeration. The number of surviving bacteria at different time points are divided by the starting population to determine the surviving proportion at each time point. The proportion of surviving MT bacteria are divided by the proportion of surviving WT bacteria to determine the fold survival (MT/WT) at each time point as depicted in Fig. 7b, Supplementary Data 8.

#### Tn-Seq experiments, fitness (W) and enrichment analyses

Six independent transposon libraries, each containing ~10,000 insertion mutants, were constructed with transposon Magellan\textsuperscript{22} in WT-T4 as described previously\textsuperscript{14,18,39,77}. Selection experiments were conducted in rich medium with glucose as a carbon source in the absence of 20 different antibiotics at a concentration that slows growth by ~30–50% (Supplementary Data 1). Libraries are grown in ~10 mL medium (wt/wt ABX), from a starting OD\textsubscript{600} of ~0.03 (~40,000 CFU/mL) up to an OD\textsubscript{600} of ~0.3–0.6 (~1.1×10\textsuperscript{8} CFU/mL), representing ~7–8 generations. Sample preparation, Illumina sequencing and fitness calculations were done as described\textsuperscript{18,39,40,60,77}. Sequencing analyses and fitness calculations are performed with analysis platform Aerobio v2.378. In short, fitness of a single mutant (W) is calculated by comparing the fold expansion of the mutant to the fold expansion of the population and is determined by the following exponential growth equation we previously developed\textsuperscript{18,39,60}:

\[
W_i = \frac{\ln(N(t_i) - d/N(t_i))}{\ln(1 - (N(t_i)/d))}
\]

where di and N(tj) are the mutant frequency at the beginning and end of the experiment respectively and d is the population expansion. All of the insertions in a specified region or gene are then used to calculate the average fitness and standard deviation of the gene knockout in question. The advantage of using this approach is that W represents the actual growth rate per generation, which makes fitness independent of time and enables comparisons between conditions. Tn-Seq fitness effects are significantly different between conditions three requirements have to be fulfilled: (1) W is calculated from at least three data points, (2) the difference in fitness between the presence and absence of antibiotic has to be larger than 15% (thus \( W_i - W_{\text{int}} = c < -0.15 \) or >0.15), and (3) the difference in fitness has to be significantly different in a one sample t-test with Bonferroni correction for multiple testing\textsuperscript{18,39,60}. Importantly, we have previously validated that a mutant’s Tn-Seq fitness is indeed directly related to its relative growth rate, which means that a mutant with for instance a Tn-Seq fitness of 0.5 (W\textsubscript{int} = 0.5) grows twice as slow as the WT. In this study, we show that fitness is also directly related to a mutant’s relative growth rate, where negative fitness indicates increased ABX sensitivity and decreased relative growth, while positive fitness indicates decreased ABX sensitivity and increased relative growth. Moreover, while Tn-Seq selection is performed under ABX pressure during growth for ~7–8 generations we find here that positive fitness in many cases also leads to increased survival, i.e., tolerance.

In multiple figures \( \Delta W (W_{\text{ABX}} - W_{\text{int}}) \) is displayed, which means that each gene’s antibiotic-specific fitness is statistically compared to baseline fitness without ABXs. \( \Delta W \) thereby indicates a gene’s antibiotic-specific fitness effect which can be categorized as: (1) Neutral, \( \Delta W = 0 \), a mutant’s relative growth is similar in the absence and presence of an ABX. Negative, \( \Delta W < 0 \), a mutant’s relative growth is significantly lower and thus grows relatively slower in the presence of an ABX; (3) Positive, \( \Delta W > 0 \), a mutant’s fitness is significantly higher and thus grows relatively faster in the presence of an ABX. For instance, if a gene knockout’s \( \Delta W = 0.25 \), it means that its relative growth rate is ~25% higher in the presence of an ABX than in its absence of the ABX.

To determine whether a particular process or pathway is specifically involved in responding to an antibiotic group, a hypergeometric test was performed to test for enrichment. The distribution of significant genes within each process was compared to the distribution of the pathways in the overall genome. A p value and Benjamini–Hochberg adjusted p value were calculated for each process and antibiotic group, where an adjusted p value below 5% is considered to identify statistically significant enrichment.

#### Determination of minimum inhibitory concentration (MIC)

MICs were determined as previously described in ref. \textsuperscript{37}. In short, ~1×10\textsuperscript{6} CFU of mid-exponential bacteria are cultured in 200 µL in 96-well plates in fresh medium containing a single antibiotic at the following concentration gradients and increments: Cefuroxim gradient 0.4–1.8 µg/mL with 0.1 µg/mL increments; Cefepime 0.0175–0.03 µg/mL with 0.0025 µg/mL increments; Gentamicin 17.39–55 µg/mL with 2.5 µg/mL increments; Meropenem 0.006–0.02 µg/mL with 0.002 µg/mL increments; ceftiofur 4.5–10 µg/mL with 0.5 µg/mL increments; vancomycin 0.12–0.32 µg/mL with 0.04 µg/mL increments. MICs were determined in triplicate and monitored on a Tecan Infinite 200 PRO plate reader or BioSpa 8 (BioTek) at 37 °C for 12 h. MIC is determined as the lowest concentration that abolishes bacterial growth (Supplementary Data 1).

#### Co-fitness network construction and SAFE analysis

A gene x condition matrix was constructed to identify correlating fitness profiles and built a co-fitness network. The co-fitness is based on the condition-timepoint performed here, supplemented with 17 conditions from van Opisen and Camilli\textsuperscript{38} (Supplementary Data 3). The additional conditions consist of sucrose, fructose, cellobose,
Cytoscape79, with the absolute correlation value network consisting of 1519 genes and 2399 edges. An edge-weighted spring used to con

Cell fractionation, TCA precipitation and western blotting

column, and Q Sepharose. Fractions were pooled and dialyzed followed by 0.22 μm filter sterilization. Protein was initially analyzed by SDS-PAGE and Western blot using an HRP-conjugated antibody against His-tag. The reaction was considered specific when the band was detected in the 1519 gels.

Cell fractionation was performed using a 1:3000 dilution of mouse-anti-His-antibody, sourced from Bio-Rad catalog# 1721019 at a concentration of 1:500. After washing, membranes were treated with secondary antibody goat anti-rabbit IgG-HRP (Bio-Rad catalog #1721019) at a concentration of 1:3000. The membranes were then treated with vancomycin (0.25 mg/kg), daptomycin (0.25 mg/kg), aSP_1505 antibody (100 μL), and control rabbit IgG. At 16 h following antibody/antibiotic treatment (24 h post challenge) mice were euthanized, and lungs and chest cavity blood were removed for quantification of bacteria. Whole lungs were washed twice in PBS, and lung tissue was subsequently homogenized in 1 mL PBS. Homogenized lung samples were centrifuged at 3000 x g, and the supernatant containing bacteria was plated onto blood agar plates for CFU titers. Mouse experiments were approved under St. Jude Children’s Research Hospital IACUC approved protocol #538 and Boston College IACUC approved protocol #2019-007-01. Mice were housed with a 12 h/12 h:dark/ light cycle. The room temperature set point was 71 degrees F (±2 degrees) and the humidity setpoint was 40%.

Peptide production. Peptide P1 (Ser-Asn-Gly-Leu-Asp-Val-Gly-Lys-Ala-Asp) and P2 (Lys-Thr-Lys-Hle-Ille-Lys) were synthesized on a preloaded Wang resin using the standard Fmoc/peptide synthesis. All coupling reactions were carried out in DMF using HBTU as the coupling reagent, 0.4 N-Methyl Morpholine in DMF as base. After each coupling, deprotection of the Fmoc group was done by using 20% piperidine in DMF. After completion of synthesis, peptides were cleaved from resin using TFA and purified using RP-HPLC. The integrity and purity of the peptides were confirmed using LC-MS.

Antibiotic accumulation. Antibiotic accumulation was determined as previously described. S. pneumoniae were grown in THY to OD 0.6. Cells were pelleted, washed twice in PBS and resuspended in 3.5 mL PBS. 1 mL of cells were incubated with 50 μM antibiotic for 10 min at 37°C. Following incubation, 800 μL of drugged cells were spun (3 min, 13,000g) through 700 μL of a 9:1 mix of AR20 and high temperature silicon oils (cooled to ~80°C), after which the supernatant of silicon oil and free compound were carefully removed. For lysis, pelleted cells were resuspended in 200 μL DI H2O and lysed via bead beating (3x 15 s at 5 m/s). Debris was pelleted (3 min, 20,000g) and 100 μL of supernatant was removed and saved. Cell debris was resuspended in the remaining 50 μL DI H2O and mixed with 200 μL methanol. Potential cell debris was pelleted again and 150 μL of the methanol extract was mixed with the 200 μL DI H2O supernatant from the previous step. The extract was pelleted one final time (10 min at 20,000g) before being filtered (0.22 μm).

Samples were acquired with a Waters Acquity M Class series UPLC system and Xevo G2 QTof tandem MS/MS with Zopyr. Hundred nanoliters of extract was separated on the Waters Acquity BEH C18 column (1.7 μm, 2.1x150 mm) at a flow rate of 0.1 mL min

In the results, the cellular fractionation of the unknown compounds were determined by the linear fit of the corresponding standard curve. Concentrations are reported as the average of three biological replicates.
(p)pGpp induction and LC/MS analysis. S. pneumoniae strains were grown at 37°C in 10 mL ThB to an OD of ~0.5. Cultures were split into 5 mL aliquots for mupirocin-induced versus untreated controls. To induce transcriptional and phenotypic stress responses and ppGpp production, mupirocin was added in a final concentration of 25 µg/mL and incubated at 37°C for 30 min. Cells were centrifuged at 6000 x g for 5 min, supernatant was discarded and cell pellets were frozen at −80°C. For LC/MS analysis cell pellets were resuspended in 2 mL cold methanol and 150 µmol of [13C6]-GTP (Sigma) was added and incubated at −80°C for 30 min. Samples were centrifuged at 4000 x g for 10 min, and the supernatant was removed and dried overnight in a Savant SpeedVac Concentrator SPD 1010 (Thermo Fisher). Samples were analyzed using a Shimadzu Prominence UFLC attached to a QTRap 4500 equipped with a Turbo V ion source (ScieX). Samples (5 µL) were injected onto a ScieQuant ZIC-C18HILIC, 3 µm, 2.1 x 150 mm column at 30°C (Millipore) using a flow rate of 0.3 mL/min. Solvent A was 25 mM ammonium acetate and Solvent B was 75% acetonitrile + 25 mM ammonium acetate. The HPLC program was the following: starting solvent mixture of 0% A/100% B, 0 to 2 min isocratic with 100% B; 2 to 4 min linear gradient to 85% B; 4 to 7 min linear gradient to 65% B; 7 to 22 min isocratic with 65% B; 22 to 25 min linear gradient to 100% B; 25 to 30 min isocratic with 100% B. The QTRap 4500 was operated in the negative mode, and the ion source parameters were: ion spray voltage, ~4500 V; curtain gas, 30 psi; temperature, 400°C; collision gas, gas medium; ion source gas 1, 20 psi; ion source gas 2, 35 psi; declustering potential, ~40 V; and collision energy, ~40 V. The MRM transitions are ppGpp, 660.1/159.0; ppGpp, 682.0/159.0, and [13C6]-GTP, 522.0/159.0. [13C6]-GTP was used as the internal standard. The system was controlled by the Analyst software 1.7 (ScieX) and analyzed with MultiQuant 3.0.2 software (ScieX). Peaks corresponding to ppGpp and ppGpp were quantified relative to the internal standard. The limit of detection for ppGpp and ppGpp is 5 pmol, and for GTP, GDP, ATP and ADP 0.05 pmol.

In vivo mouse competition experiment w/o antibiotics. In vivo competition experiments were essentially performed as previously described (van Opijnen and Camilli 2012). Specifically, groups of at least 12 outbred 4–6-week-old Swiss Webster mice (Taconic Inc.), were anesthetized by isoflurane inhalation and challenged intranasally with 50 µL, ~1.5 x 10⁷ CFU, bacterial suspension in 1×SSC. Mice were euthanized by CO₂ asphyxiation at 4000 × g for 10 min. First, 90% of the gene. For each gene set the number of strains with at least one stop codon in the gene set were recorded, as well as the total number of stop codons in all genes in a set. To test for differences in a set, the exact test was performed. Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Sequencing data generated in this study is available at the Short Read Archive (BioProject accession number PRJNA750088). Tn-Seq fitness data used across the manuscript can be found in Supplementary Data 2. Growth and tolerance data can be found in Supplementary Data 8 and Purine Metabolism data in Supplementary Data 9. Gene annotation information was obtained from PATRIC (https://www.patricbrc.org/), GenomeNet (https://www.genome.jp) and UniProt (https://www.uniprot.org/). Gene sets were assembled from sequences obtained from PATRIC. Source data are provided with this paper.

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Author contributions
T.v.O. devised the study and wrote the manuscript. E.R., B.S., L.M.N.R., F.R, A.T.N., S.I.W., B.J., N.B, K.L., J.G., M.F., S.M.R., R.E.L., C.O.R., J.W.R., and T.v.O. performed wet-lab experiments, data collection and interpretation. D.L. and T.v.O. performed Tn-Seq data analysis, pathway and network construction, analysis and interpretation. J.W.R. contributed to key conceptual ideas. All authors contributed to manuscript editing and approved the final paper.

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The authors declare no competing interests.

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