Evolution of high-level resistance during low-level antibiotic exposure

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It has become increasingly clear that low levels of antibiotics present in many environments can select for resistant bacteria, yet the evolutionary pathways for resistance development during exposure to low amounts of antibiotics remain poorly defined. Here we show that *Salmonella enterica* exposed to sub-MIC levels of streptomycin evolved high-level resistance via novel mechanisms that are different from those observed during lethal selections. During lethal selection only *rpsL* mutations are found, whereas at sub-MIC selection resistance is generated by several small-effect resistance mutations that combined confer high-level resistance via three different mechanisms: (i) alteration of the ribosomal RNA target (*gidB* mutations), (ii) reduction in aminoglycoside uptake (*cyoB, nuoG, and trkH* mutations), and (iii) induction of the aminoglycoside-modifying enzyme *AadA* (*znuA* mutations). These results demonstrate how the strength of the selective pressure influences evolutionary trajectories and that even weak selective pressures can cause evolution of high-level resistance.

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rrespective of whether antibiotics are used to treat infections in humans or animals, for growth promotion in animals, aquaculture, or plant production, a substantial fraction of these antibiotics will ultimately end up in the environment. Thus, there are many environments such as wastewater, sludge, soil, and river water where bacteria are exposed for long periods of time to low concentrations of polluting antibiotics that are present because of anthropogenic influences. Furthermore, low antibiotic concentrations (below the minimal inhibitory concentration, MIC) might be present in certain human/animal body compartments and tissues during therapeutic or growth promotion use. Previous studies showed that low levels of antibiotics (sub-MIC) can enrich for pre-existing resistant mutants in a bacterial population, indicating that certain antibiotics, disinfectants, and heavy metals could contribute to resistance evolution at concentrations that are several hundred-fold below the MIC.

While many studies have examined the genetics of mutational antibiotic resistance selected at high levels (>MIC) of antibiotics, less is known about the effects of long-term exposure to low levels (<MIC) of antibiotics. When susceptible bacteria are exposed to antibiotic concentrations above the MIC they will die or stop growing, and only bacteria where resistance mutations were present prior to antibiotic exposure will be able to grow. In contrast, at sub-MIC concentrations of antibiotics the bacteria can still grow while they are under selection, generating a potentially different trajectory of evolution with progressive increase in resistance through the step-wise accumulation of resistance mutations with individually smaller effects. During selection at high concentrations of streptomycin the most common resistance mutations are target alteration mutations in the gene rpsL, encoding ribosomal protein S12, that confer high-level resistance >1024 mg L\(^{-1}\). Mutations in the electron transport chain that confer a small colony variant (SCV) phenotype can also cause a moderate increase in resistance (16–96 mg L\(^{-1}\)), which is often linked to a severe reduction in growth rate.

In this work, we investigated the evolution and genetics of antibiotic resistance selected during long-term exposure to streptomycin at sub-MIC level of the drug in Salmonella enterica, an important bacterial pathogen and model system for studies of antibiotic resistance. Results show that resistance evolution below the MIC of streptomycin occurs via different mechanisms compared to lethal selection and that high-level resistance can evolve also when bacteria are exposed to low antibiotic concentrations (sub-MIC).

**Results**

**Mutant selection above MIC.** Many previous studies have shown that rpsL mutations are the major type of mutants found at selection above the MIC. We reconfirmed these results and showed that when 10 independent cultures of susceptible Salmonella enterica serovar Typhimurium LT2 strain (designated S. typhimurium throughout the text) were selected on Mueller–Hinton (MH) agar for streptomycin resistance at 200 mg L\(^{-1}\) of streptomycin (50× above the MIC), 10/10 mutants had mutations in rpsL (amino acid substitutions: six K42R, one K42N, one K42T, and two K87R) that conferred the resistance. Whole-genome sequencing of six independent isolates confirmed that mutants selected on high streptomycin concentrations on agar plates had only rpsL mutations. Furthermore, we also performed a serial passage experiment (100 generations) in liquid MH containing 200 mg L\(^{-1}\) streptomycin. Whole-genome sequencing of five populations showed that the only resistance conferring mutations present in them were rpsL mutations (K42R). Thus, for 11 independent selections at high streptomycin levels only rpsL mutants were selected.

**Mutant selection below MIC.** To study evolution of antibiotic resistance in a susceptible bacterial population below MIC, 20 independent lineages of the streptomycin susceptible wild-type S. typhimurium were serially passaged for 900 generations in MH medium containing 1 mg L\(^{-1}\) of streptomycin, corresponding to 1/4 of the MIC of the susceptible wild type. The concentration of streptomycin used causes an approximately 3% reduction in competitive growth rate of the susceptible wild type and was chosen to provide a sub-MIC selection. This estimation was based on previous work, where in a serial passage competition experiment 1 mg L\(^{-1}\) streptomycin balances the 3% fitness cost conferred by an rpsL (K42R) mutation. Serial passage occurred every 24 h by transfer of 1 µl of overnight culture (5 × 10\(^9\) cells/ml) to 1 ml of culture medium, generating a bottleneck of 5 × 10\(^8\) cells during transfer. After serial passage, bacteria were plated on MH agar plates with different concentrations of streptomycin (8, 16, 32, 64, 96, 128, 192, and 256 mg L\(^{-1}\)) to estimate the frequency of cells with different resistance levels. The populations were heterogeneous with regard to resistance and several of the lineages contained subpopulations (approximately 0.1–1% of the cells) with high levels of resistance (MIC of streptomycin >96 mg L\(^{-1}\)). Clones with increased resistance were single-colony isolated from six independent lineages, and these purified clones were further analyzed. The MICs of streptomycin varied between 64 mg L\(^{-1}\) and >1024 mg L\(^{-1}\) depending on the mutant. Four clones (obtained from four different lineages) with the highest streptomycin resistances (>192 mg L\(^{-1}\)) were investigated further. In control experiments, where bacteria were serially passaged for 1000 generations in the absence of streptomycin, no streptomycin-resistant mutants were found.

The spectra of mutations at sub-MIC and >MIC selection differ. To identify the mutations responsible for the increased resistance to streptomycin, the four clones with the highest resistance levels were whole-genome sequenced, and their sequences were compared to that of the ancestral strain to identify potential point mutations and structural rearrangements (deletions, duplications, insertions, and inversions). The results showed that all four strains had mutator phenotypes, with roughly 100 different point mutations per genome (Supplementary Table 1). The mutator phenotypes are likely to be caused by mutations in the DNA mismatch repair system since the strains had mutations in the mutS/LH genes.

To identify which mutations contributed to the high-level streptomycin resistance, we first examined if there were mutations present in the serially passaged strains that have previously been implicated in resistance. As shown by our present data and previous studies, during selection for streptomycin resistance at high levels of streptomycin, mainly mutations in the gene rpsL, encoding the 30S ribosomal subunit protein S12, are found. However, none of the clones isolated here from serial passage at sub-MIC concentrations had rpsL mutations. Instead, all of them had loss-of-function mutations in the gidB gene, encoding a 16S rRNA methyltransferase that has been linked to low levels of streptomycin resistance in Salmonella and Mycobacterium tuberculosis. In addition, the strain with the highest streptomycin resistance also carried a mutation in the gene trkH encoding a potassium symporter, a gene previously shown to be involved in aminoglycoside resistance in Escherichia coli.

To elucidate which additional mutations might be involved in the high-level resistance, we identified genes that were mutated in...
more than one lineage with the assumption that repeated occurrence of mutations in a specific gene is likely to reflect a response to the antibiotic selection, rather than fixation of a random non-selected mutation (however, it should be noted that media adaptation mutations could also show repeated appearance in independent lineages, see below). Thus, besides the $gidB$ and $trkH$ mutations the strains with the highest streptomycin resistances (Table 1 and Supplementary Table 1) had also accumulated mutations in genes involved in the respiratory electron transport chain, such as $nuoG$ and $nuoE$, encoding subunits of NADH dehydrogenase and cyoB, encoding the cytochrome c oxidase subunit I. Two of the strains also carried mutations in the $znuAB$ genes encoding proteins involved in zinc uptake. This generated a list of five candidate mutations: $gidB$, $trkH$, $nuoG$, cyoB, and $znuA$ that were examined further.

**Combination of five mutations generated high-level resistance.** The MIC of streptomycin for the *S. typhimurium* strain (DA6192) used as a starting ancestral strain for the sub-MIC cycling was 4 mg L$^{-1}$ and the isolated resistant mutants had MIC values ranging from 192 to $>$1024 mg L$^{-1}$ (Table 1). To examine how the putative resistance mutations contributed to the highest level of resistance ($>$1024 mg L$^{-1}$), we reconstructed all possible combinations of the five candidate genes: $gidB$, cyoB, $nuoG$, $trkH$, and $znuA$ (identified as described above), generating 32 different mutants (Fig. 1, Table 2). Of the five reconstructed single mutants only the mutation in the gene $gidB$ conferred a significant increase in resistance to streptomycin. Thus, the loss-of-function mutation $gidB$ (Q169*) (where * represents a stop codon) gave a MIC of 32 mg L$^{-1}$ for streptomycin, similar to previously reported values25. In contrast, the other single mutants gave no ($trkH$), 1.5-fold ($znuA$), or 2-fold (cyoB, $nuoG$) increases in resistance (Table 2 and Fig. 1). By combining several of the mutations generating all potential double, triple, quadruple, and quintuple mutants, a step-wise increase in resistance was observed, reaching up to $>$1024 mg L$^{-1}$. Thus, adding mutations in the respiratory chain to a $gidB$ (Q169*) genetic background increased the resistance further, where mutations in cyoB (G283D) or $nuoG$ (D264G) individually increased the MIC to 96 mg L$^{-1}$, while the combination of both gave a MIC of 256 mg L$^{-1}$ (Table 2 and Fig. 1). Reconstitution of the selected $znuA$ (S19fs) (where “fs” represents a frame shift) mutation in a genetic background of $gidB$ (Q169*), cyoB (G283D), and $nuoG$ (D264G) increased the MIC even further to 768 mg L$^{-1}$ (Table 2 and Fig. 1). Finally, the addition of the $trkH$ (T99A) mutation increased the resistance even further to a MIC of $>$1024 mg L$^{-1}$. A comparison of the resistance levels of the single mutants with the higher-order mutants showed that there were strong epistatic interactions between the mutations to generate the high-level resistance (Fig. 2 and Supplementary Table 2). Thus, the expected additive resistance (without epistasis) is a 36-fold increase in MIC in the quintuple mutant compared to the wild type, while the observed increase was $>$220-fold (Fig. 2a).

Other types of mutations that were found in several of the sequenced clones were mutations in genes in the maldose operon (*malT* and *malF*) and genes encoding tRNAs. The *mal* mutations had no effect on resistance and are likely to be media adaptation mutations31,32. Similarly, mutations in genes involved in flagellar biosynthesis (*flgC*, *flgK*, *filN*, and *flf*) were also seen in MH media adapted *Salmonella* strains, whereas the role of the tRNA mutations is unknown.

**The role of the aadA gene and the stringent response in resistance.** Previous studies showed that activation of the cryptic aminoglycoside resistance gene *aadA* in *S. typhimurium* is ppGpp-dependent, and that upregulation of this gene results in increased resistance to streptomycin25. The *aadA* gene encodes an aminoglycoside adenyl transferase which inactivates the drug

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**Table 1 Isolated resistant mutants**

| Strain | Genotype | Streptomycin MIC (mg L$^{-1}$) | Reference |
|--------|----------|-------------------------------|-----------|
| DA6192 | Wild type (parent) | 4 | Strain collection |
| DA23868* | Mutant isolated by plating serial passage population on 256 mg L$^{-1}$ streptomycin and picking a single colony (for complete genotype, see Supplementary Table 1) | 512 | This study |
| DA23869* | Mutant isolated by plating serial passage population on 256 mg L$^{-1}$ streptomycin and picking a single colony (for complete genotype, see Supplementary Table 1) | $>$1024 | This study |
| DA23872* | Mutant isolated by plating serial passage population on 192 mg L$^{-1}$ streptomycin and picking a single colony (for complete genotype, see Supplementary Table 1) | 512 | This study |
| DA23874* | Mutant isolated by plating serial passage population on 128 mg L$^{-1}$ streptomycin and picking a single colony (for complete genotype, see Supplementary Table 1) | 192 | This study |
| DA23877* | Mutant isolated by plating serial passage population on 96 mg L$^{-1}$ streptomycin and picking a single colony (for complete genotype, see Supplementary Table 1) | 96 | This study |
| DA23879* | Mutant isolated by plating serial passage population on 96 mg L$^{-1}$ streptomycin and picking a single colony (for complete genotype, see Supplementary Table 1) | 64 | This study |
| DA25308 | DA23868, aadA::cat | 4 | This study |
| DA25310 | DA23869, aadA::cat | 16 | This study |
| DA25312 | DA23872, aadA::cat | 8 | This study |
| DA25314 | DA23874, aadA::cat | 8 | This study |
| DA26068 | DA23868, relA::Tn10dTet | 512 | This study |
| DA26070 | DA23869, relA::Tn10dTet | 384 | This study |
| DA26072 | DA23872, relA::Tn10dTet | 128 | This study |
| DA26074 | DA23874, relA::Tn10dTet | 128 | This study |
| DA27220 | DA23868, relA::Tn10dTet, ΔspoT::cat | 192 | This study |
| DA27222 | DA23869, relA::Tn10dTet, ΔspoT::cat | 256 | This study |
| DA27224 | DA23872, relA::Tn10dTet, ΔspoT::cat | 96 | This study |
| DA27226 | DA23874, relA::Tn10dTet, ΔspoT::cat | 48 | This study |

Streptomycin resistance of *S. typhimurium* clones isolated from serial passage and the effects of aadA and relA, spoT inactivation mutations on resistance. MIC values presented are the median values of 2-5 Etests from biological replicates.

* Note that the four whole-genome sequenced clones contained many mutations (see Supplementary Table 1 for list of all mutations)
by covalent addition of an adenyllyl group. To examine the potential importance of adaA in the high-level streptomycin resistance observed in this study, we deleted the adaA gene in four of the clones isolated from the serial passage experiment (Table 1), as well as the reconstructed mutants (Table 2). This deletion resulted in a strong reduction of resistance for all mutants (Table 1), suggesting that the adaA gene has a central role in streptomycin resistance evolution at sub-MIC concentrations. Since the adaA gene is regulated by the stringent response, we also deleted the genes responsible for ppGpp synthesis, relA and spoT, and this also lowered the resistance levels of the majority of the strains (Tables 1 and 2). These findings demonstrate that the high-level streptomycin resistance of the strains require the ability to induce stringent response and presence of the AadA protein.

**Loss of znuA increase adaA transcript levels.** Since high-level resistance requires the presence of the AadA protein (Tables 1 and 2), we determined if the increase in resistance in the reconstructed mutants was due to elevated adaA expression. We isolated total RNA at late exponential phase from all single mutants and two double, two triple, two quadruple, and the quintuple mutant and measured adaA transcript levels using qRT-PCR (Fig. 3). We observed a 7-fold increase in adaA expression in the znuA single mutant, while all other single mutants showed no significant increase. In accordance with this, all mutants with more than one mutation that also included znuA showed increased adaA expression levels to the same extent as the znuA single mutant. The two reconstructed clones containing multiple mutations without znuA (the double and triple mutants gidB, trkH, and gidB, nuoG, trkH) showed wild-type levels of adaA transcript. Thus, the znuA mutations (but not gidB, trkH, cyoB, or nuoG mutations) confer increased streptomycin resistance by causing adaA upregulation and inactivation of streptomycin via adenyllylation. To test whether loss of ZnuA rather than the production of a frameshifted protein is causing the observed phenotype we constructed a deletion of the znuA reading frame in a wild-type background as well as in a quadruple mutant consisting of gidB, nuoG, cyoB, and trkH (Fig. 3). In both cases the deletion of znuA resulted in a 11-fold upregulation of adaA transcript levels, showing that loss of ZnuA is responsible for the observed phenotype. In addition, the upregulation of adaA due to loss of ZnuA was completely restored if the cells were grown in the presence of excess zinc, demonstrating that it is zinc starvation and not lack of the ZnuA protein per se that caused upregulation of AadA.

**cyoB, nuoG, and trkH mutations reduce streptomycin uptake.** Since it is known that proton motive force is needed for streptomycin uptake,33-36 and that the cyoB, nuoG, and trkH mutations are likely to impair electron transport,30,37 we used radioactive streptomycin to measure if uptake was reduced in the mutants. Results show (Fig. 4) that one of the single mutants (cyoB) and the triple mutant (trkH, nuoG, cyoB) had a significant reduction in intracellular levels of tritiated dihydrostreptomycin, supporting the notion that these mutations confer increased resistance by impairing drug uptake. While the observed decrease in intracellular streptomycin is only moderate, streptomycin uptake and resistance level does not necessarily correlate in a linear and proportional way, and a small effect on uptake could conceivably have a large effect on resistance. Additionally, the reduction in streptomycin uptake is likely to be underestimated.
since the background signal in all strains might partly result from extracellularly bound streptomycin.

**Table 2 Reconstructed strains**

| Strain   | gidB | trkH | cyoB | nuoG | znuA | Streptomycin MIC (mg L⁻¹) | Streptomycin MIC with ΔaadA | Streptomycin MIC with relA::Tn10 spoT::cat |
|----------|------|------|------|------|------|---------------------------|-----------------|-----------------------------------|
| DA6192   | wt   | wt   | wt   | wt   | wt   | 4                         | 2               | 1.5                               |
| DA40134  | Q169*| T99A | G283D| D264G| S19fs| 32                        | 4               | 16                                |
| DA39332  |      |      | G283D|      |      | 8                         | 4               | 8                                 |
| DA40118  |      | T99A |      |      |      | 8                         | 2               | 1.5                               |
| DA39336  |      |      | G283D|      |      | 6                         | 2               | 6                                 |
| DA40124  | T99A |      |      |      |      | 3                         | 2               | 2                                 |
| DA40138  | T99A | G283D| D264G|      |      | 96                        | 8               | 48                                |
| DA40471  | Q169*| G283D| D264G|      |      | 96                        | 12              | 48                                |
| DA40120  |      | G283D| D264G|      |      | 16                        | 8               | 16                                |
| DA40142  | Q169*|      |      |      |      | 64                        | 4               | 24                                |
| DA40130  |      | D264G|      |      |      | 16                        | 4               | 8                                 |
| DA40122  |      | G283D|      |      |      | 12                        | 6               | 6                                 |
| DA40473  | Q169*|      |      |      |      | 24                        | 6               | 48                                |
| DA40126  | T99A | D264G|      |      |      | 6                         | 4               | 6                                 |
| DA40482  | T99A | G283D|      |      |      | 12                        | 6               | 12                                |
| DA40128  | T99A |      |      |      |      | 4                         | 2               | 3                                 |
| DA40379  | Q169*| G283D| D264G|      |      | 256                       | 16              | 96                                |
| DA40387  | Q169*| G283D| D264G|      |      | 192                       | 8               | 96                                |
| DA40383  | Q169*| G283D|      |      |      | 128                       | 8               | 96                                |
| DA40389  |      | G283D| D264G|      |      | 32                        | 12              | 16                                |
| DA40475  | Q169*| T99A | D264G|      |      | 192                       | 12              | 16                                |
| DA40483  | Q169*| T99A | G283D| D264G|      | 96                        | 16              | 48                                |
| DA40378  | T99A | G283D| D264G|      |      | 16                        | 12              | 16                                |
| DA40477  | Q169*| T99A |      |      |      | 96                        | 4               | 24                                |
| DA40385  | T99A | D264G|      |      |      | 12                        | 4               | 12                                |
| DA40381  | T99A | G283D|      |      |      | 12                        | 6               | 12                                |
| DA40467  | Q169*| G283D| D264G|      |      | 768                       | 24              | 256                               |
| DA40461  | Q169*| T99A | G283D| D264G|      | 192                       | 24              | 96                                |
| DA40465  | Q169*| T99A | G283D| D264G|      | 192                       | 8               | 96                                |
| DA40463  | Q169*| T99A | G283D| D264G|      | 128                       | 12              | 128                               |
| DA40469  | Q169*| T99A | G283D|      |      | 32                        | 6               | 12                                |
| DA40479  | Q169*| T99A | G283D|      |      | 1024                      | 16              | 256                               |

Genotypes and streptomycin resistances of reconstructed strains and the effects of aadA and relA, spoT inactivation on resistance. The symbol “*” represents a stop codon, while “fs” represents a frame-shift mutation. MIC values presented are the median values of 2–5 Etests from biological replicates.

Modeling the step-wise selection of five mutations. To examine the dynamics of the step-wise enrichment of several mutations in one clone and determine under which conditions five step-wise mutations could accumulate in 900 generations under this weak selection, we modeled the selection process (for details, see Methods). In these calculations, we applied the experimentally determined population sizes and generations of growth used during serial passage. We also assumed that all selected mutations contribute independently to resistance/fitness and that each mutation has the same selection coefficient $s = 0.006$, such that five of them together give a 3% growth advantage in the presence of the antibiotic (during the selection the used concentration of streptomycin at ¼ MIC reduced the growth rate of the susceptible wild-type by 3%). Furthermore, a mutator mutation is assumed to occur with the same rate, $u$, as each of the five selected resistance mutations (which are all loss-of-function mutations). In addition, it is assumed (as also observed experimentally) that the high-level-resistant mutants reached a frequency of 1% in the evolving population after 900 generations of growth. Results show that without mutators, 1% penetration after 900 generations can be achieved only with an unrealistically high mutation rate, $u = 10^{-3}$, for each individual resistance mutation. In contrast, when mutators with mutation rate $v$ are present, the mutant with five resistance mutations can reach 1% penetration in 900 generations within a realistic parameter range where mutation rate $u$ varies ca. 10-fold between $5 \times 10^{-7}$ and $4 \times 10^{-6}$ and the ratio $v/u$ also varies 10-fold between ca. $10^2$ and $10^3$ (Table 3, Fig. 5). Thus, even with a weak selection ($s = 0.03$) and only 900 generations of evolution, it is possible to step-wise accumulate five resistance mutations provided mutator clones appear early during serial passage.

**Discussion**

In this study, we exposed susceptible wild-type bacteria to constant low levels of antibiotics (4-fold below the MIC, 1 mg L⁻¹) for 900 generations, and found that high-level resistance (up to >1024 mg L⁻¹) evolved in several independent lineages. By whole-genome sequencing and genetic reconstructions, we identified five causative resistance mutations of individual clones. The mutations had individually small effects, but when combined in a wild-type background they resulted in high-level resistance due to strong positive epistasis between certain mutations (Fig. 2, Supplementary Table 2). Since the evolved strains contained more mutations than the five that we studied in detail it is possible that other mutations also contributed to the resistance and fitness phenotypes.

An important question is how consecutive mutations in five different genes can accumulate over only 900 generations of selection with the weak selection used. Our modeling results show that with a 3% reduction in growth rate, a mutation rate for each consecutive mutation of around 3.5–5 $\times 10^{-4}$ per cell per generation is required for the step-wise accumulation of five consecutive mutations in 900 generations with the serial passage.
population sizes used and when the quintuple mutant constitutes 1% of the total population. All the resistance phenotypes are caused by loss-of-function mutations in the five different target genes and from previous work it is known that the average loss-of-function rate in a gene in a wild-type *E. coli* S. enterica is approximately $10^{-6}$ per cell per generation. In addition, all the examined clones are mutators, and the likely explanation for the mutator phenotype is that step-wise selection of several independent resistance mutations enriches for the mutators. It is well described that mutator alleles can rapidly become enriched in asexual populations during step-wise adaptation by second-order selection because they remain associated with the rare favorable resistance mutations they generate. Importantly, the mutators (MutHLS mismatch repair system) found in this study exhibit an approximately 150-fold increase in mutation rate as compared to the wild type (Supplementary Table 3), which brings the expected loss-of-function for each individual gene to $10^{-4}$ per cell per generation, which is near the theoretically required mutation rate. We also attempted to experimentally determine which of the many possible routes to the high-level-resistant quintuple mutant (Fig. 1) occurred in the different lineages and how fitness changed in the evolving populations over time. However, this was not possible due to the fact that all of these strains are mutators and as a result the heterogeneity in each population is very high, making a time series analysis by deep sequencing of the evolving population difficult. In addition, the high heterogeneity in combination with the mutator phenotype also preclude us from making accurate measurements of small fitness differences (<1%) by competition since the competitor strains would rapidly acquire media adaptation mutations that alter their fitness.

How is resistance conferred by the identified mutations? Based on the experiments presented here and previous studies, we propose that the high-level resistance is caused by the combined effect of three different mechanisms: (i) rRNA target alteration (due to *gidB* mutations), (ii) reduced aminoglycoside uptake (due to *cyoB*, *nuoG*, and *trkH* mutations), and (iii) induction of the aminoglycoside modifying enzyme AadA (due to *znuA* mutations) (Fig. 6). With regard to the first mechanism, all of the strains had loss-of-function mutations in the *gidB* gene, encoding a 16S rRNA methyltransferase. This enzyme methylates the 530 loop of 16S rRNA and if the modification is absent streptomycin binding to the ribosome is reduced. Mutations in this gene have been reported to confer a moderate increase resistance to streptomycin in several species of bacteria, including *S. enterica* and *M. tuberculosis*.

The second mechanism includes mutations that are located in genes encoding proteins involved in the electron transport chain, such as genes encoding subunits of NADH dehydrogenase (*nuoG*) and cytochrome o oxidase (*cyoB*). Aminoglycosides are cationic molecules, and they are believed to first penetrate the outer membrane through porins, and then cross the cytoplasmic membrane in a process that is dependent on electron transport through quinones and a sufficiently high membrane potential. The mutations we observed in the respiratory chain (*cyoB* and *nuoG*) could potentially reduce electron transport and the membrane potential, and as result reduce streptomycin uptake. Similar mutations have also previously been found in streptomycin-resistant SCVs of *Salmonella*, but while the SCVs have a very slow growing phenotype, the mutants selected in this study at sub-MIC levels of streptomycin have higher growth rates (Supplementary Table 3). Besides genes involved directly in the respiratory chain, the mutant with the highest level of streptomycin resistance also had a mutation in the potassium symporter TrkH. The gene encoding TrkH has previously been linked to streptomycin resistance, probably by affecting the membrane electrochemical potential. Uptake assays with radiolabeled dihydrostreptomycin showed that, as expected, the *cyoB*, *nuoG*, and *trkH* mutants were impaired in drug accumulation, providing a conceivable explanation for how these mutations confer increased resistance (Fig. 4).

The third mechanism of resistance, enzymatic modification of streptomycin, is dependent on the expression of the cryptic aminoglycoside resistance gene *aadA*. Previous studies of *Salmonella* SCVs have shown that the *aadA* gene can be upregulated through the stringent response, a stress response system that uses the global regulator ppGpp to control the distribution of resources between synthesis of ribosomes when nutrients are abundant, and the increased synthesis of metabolic enzymes during amino acid starvation. Deleting the *aadA* gene in the...
mutants (both original and reconstructed) lowered resistance by 1.5-fold to 100-fold (Tables 1 and 2). However, after deletion of relA and spoT, which removes any ppGpp-dependent regulation\textsuperscript{45}, strains carrying mutations in gidB, cyoB, nuoG, and/or trkH still show increased resistance to streptomycin compared to a relA, spoT-deficient wild-type strain indicating that their effect on resistance is not dependent on stringent response but on target modification via gidB and reduced uptake via cyoB, nuoG, and trkH. Only the effect of znuA mutations seemed to be abolished in a relA, spoT-deficient background (Table 2). In addition, we observed that the mutation in znuA alone was responsible for elevated aadA expression levels (Fig. 3). The periplasmic zinc binding protein ZnuA is part of the ZnuABC system. This system is of particular importance under zinc-limiting conditions, ensuring the high-affinity uptake of Zn\textsuperscript{2+}\textsuperscript{46}. Despite the fact that this system is repressed in the presence of adequate concentrations of zinc\textsuperscript{47} it is likely that basal levels are still expressed even during growth in rich medium like MH broth. Loss-of-function mutations in znuA are therefore likely to disrupt zinc homeostasis, an ion with a central role in essential cellular enzymes and processes\textsuperscript{48}, and cause activation of cellular responses that subsequently could alter gene expression patterns. To determine if the observed upregulation of aadA in znuA-deficient mutants is mediated via increased ppGpp levels we measured the transcript levels of iraP, which is known to be positively regulated by elevated ppGpp levels\textsuperscript{49}, in a subset of mutants. None of the tested strains showed an increase in iraP transcript levels indicating a ppGpp-independent mechanism even for znuA mutants. Furthermore, ppGpp levels were not consistently increased in the znuA mutants (Supplementary Fig. 1). The transcriptional changes caused by ppGpp are often augmented by the transcription factor DksA\textsuperscript{50–52}. Since this protein contains a central Cys4 Zn-finger motif that is likely to be essential for correct folding and functioning\textsuperscript{53,54}, we hypothesized that the observed effect of zinc depletion on aadA upregulation might be caused by the loss of functional DksA. However, deletion of dksA in a wild-type background did not cause upregulation of aadA. Additionally, the introduction of a zinc-independent dksA paralog from Pseudomonas aeruginosa\textsuperscript{55} in a znuA mutant did not restore wild-type levels (Supplementary Fig. 2), showing that misfolding...
of DksA under zinc starvation is not responsible for the observed phenotypes. While the exact mechanism of aada upregulation via znuA-mediated zinc-depletion remains unclear, our results suggest that it is not mediated via ppGpp or DksA, but requires functional RelA and SpoT, possibly due to a dominant regulatory effect exerted by these proteins.

What are the general implications of this work? First, and most importantly, our results demonstrate that very weak and constant selective pressures of antibiotics can result in the evolution of high-level resistance. Thus, an implication from these results is that there is no necessary correlation between strength of selection and level of resistance evolved, and as a consequence it is conceivable that the low antibiotic levels present in many environments could drive the evolution of clinically relevant high-level resistance. Second, our data show that mutational spectra can differ strongly between different selective strengths. Thus, at lethal selections only rpsL mutations are found, whereas at sub-MIC selections a different set of mutations are selected. A likely explanation for this difference is that at >MIC levels of drug only rpsL mutations provide a sufficiently high level of resistance to be enriched, whereas at low concentrations the more common mutations in the genes identified here are preferentially observed (even though rpsL mutations occur but at a much lower rate). Third, these results show that the high-level resistance can result from many different mutations of small individual effect that show positive epistasis. Thus, among the five genes examined only mutations in the gidB gene confer measurable resistance by itself, whereas when the five mutant genes were combined resistance reached very high levels. Furthermore, a majority of these mutations were in genes that are not typical resistance genes (i.e., cyoB, nuoG, trkH, and znuA). This finding raises the question if a substantial “dark matter” of unknown resistance mutations for other classes of antibiotics exists in clinical isolates that apart from the generally easily identified bona fide resistance genes/mutations may be involved in resistance evolution and make a significant contribution to the clinical resistance phenotype.

Thus, it is conceivable that the initial selection for common mutations of small effect could act as a stepping stone for the subsequent occurrence of a rarer large effect genetic event (e.g., horizontal gene transfer of a bona fide resistance gene) and thereby act as a driver of resistance evolution. Also, considering that individually the mutations in cyoB, nuoG, trkH, and znuA conferred no/minor increases in resistance, these types of atypical resistance mutations would be difficult to identify in clinical isolates using standard genetic procedures and reconstruction. That is, transfer of DNA from a resistant strain by transformation/transduction to a susceptible strain to identify potential resistance mutations is unlikely to work since the individual mutations except for gidB had minor effects on resistance. Instead, the clinical identification of these types of mutations would first require laboratory selection and reconstitution experiments (as done here) for identification, and then a subsequent comparative analysis of susceptible and resistant clinical isolates to search for their potential presence in resistant isolates. Finally, it is worthwhile noting that the potential occurrence of atypical resistance mutations in clinical isolates would make sequence-based in silico predictions of antibiotic resistance more challenging.

**Table 3 Penetration of five mutations Fo5 + Fm5 after 900 generations**

| v/u | 90  | 125  | 203  | 285  | 400  | 460  | 1055 |
|-----|-----|------|------|------|------|------|------|
| 5×10^{-7} | 5×10^{-8} | 2.5×10^{-7} | 2.8×10^{-6} | 1.5×10^{-5} | 8.3×10^{-5} | 1.7×10^{-4} | 0.010 |
| 1×10^{-6} | 3×10^{-6} | 1.6×10^{-5} | 1.8×10^{-4} | 9.4×10^{-4} | 5×10^{-3} | 0.010 | 0.27 |
| 2×10^{-6} | 1.9×10^{-4} | 9.5×10^{-4} | 0.010 | 0.048 | 0.17 | 0.24 | 0.65 |
| 3×10^{-6} | 0.002 | 0.010 | 0.083 | 0.24 | 0.43 | 0.5 | 0.76 |
| 4×10^{-6} | 0.010 | 0.045 | 0.23 | 0.42 | 0.57 | 0.62 | 0.81 |

Mutations in a non-mutator occur with the rate u, while the mutator cells have the mutation rate v. In this calculation, the mutation rate u has been varied between 5×10^{-7} and 4×10^{-6} and the ratio u/v between ca. 10^7 and 10^8. In these ranges, a 1% penetration of the mutant with five resistance mutations (indicated in bold) after 900 generations can be reached with a mutator rate v of ca. 0.002-0.004.

**Methods**

**Strains and growth conditions.** All strains used in this study were derived from Salmonella enterica serovar Typhimurium LT2 (designated S. typhimurium in the text) and are listed in Tables 1 and 2, and Supplementary Tables 4 and 5. The liquid and solid media used for bacterial growth were MH broth (Becton Dickinson, MD, USA), MH agar (MH broth supplemented with 1.5% agar), and Luria-Bertani (LB) agar (Sigma-Aldrich, MO, USA). Strains were grown at 37°C, and liquid cultures were aerated by shaking.

**Selection for resistance above MIC.** Selection of S. typhimurium mutants with decreased susceptibility to streptomycin was performed by plating 10 independent cultures (each inoculated with 10^7 cells) on agar plates containing 200 µg L\(^{-1}\) streptomycin (Sigma-Aldrich). Approximately 10^10 cfu from each culture were plated. The mutants (one per independent culture) were randomly picked after 24 h of incubation at 37°C and re-isolated on plates with the same concentration of streptomycin. Cultures of isolated clones were grown over night at 37°C in MH broth without selection and frozen in –80°C with 10% sterile DMSO. The rpsL gene in the mutants was PCR amplified with primers binding upstream and downstream of the coding region using DreamTaq DNA polymerase (Thermo Scientific). Populations of S. typhimurium were also evolved in presence of high levels of streptomycin in liquid medium by passing 10 lineages of the wild-type strain DA6192 in MH medium with 200 µg L\(^{-1}\) streptomycin. The lineages were started from overnight cultures of independent colonies grown in MH without selection. The evolution experiment was initiated with an inoculum size of approximately 10^10 cells. The cultures were serially passaged by 1000-fold dilutions in 1 ml batch cultures every 24 h, and after 100 generations of cycling the cultures were frozen in –80°C with 10% sterile DMSO. Six mutants selected on solid media and five populations evolved in liquid media were whole-genome sequenced to identify possible streptomycin resistance mutations outside of rpsL (Supplementary Table 4).

**Laboratory evolution of resistance at sub-MIC.** The evolution of de novo resistance has been described previously. Briefly, bacteria were grown in MH medium with 1 µg L\(^{-1}\) of streptomycin for 24 h at 37°C with shaking and then 1 µl culture was transferred generating a maximum and minimum population of 5×10^7 cells and 5×10^3 ml^{-1}, respectively, during each step of the serial passage. After 900 generations (approximately 90 serial passages) of cycling, 10^7 cells were plated on LB agar containing different concentrations of streptomycin (8, 16, 32, 64, 96, 128, 192, and 256 µg L\(^{-1}\)), and 2–3 resistant clones were isolated from each lineage where they represented a fraction of the population of approximately 1% (Supplementary Table 5). The isolated clones were frozen in –80°C in 10% DMSO, and the evolving lineages were frozen every 100 generations.

**Whole-genome sequencing.** Genomic DNA (gDNA) was extracted from 4 ml of overnight cultures of the mutants isolated after 900 generations of growth in 1 µg L\(^{-1}\) of streptomycin using the Genomic-tip 100/G columns and the Genomic DNA Buffer Set (Qiagen, The Netherlands), according to the manufacturer’s instructions. Quality of gDNA was examined using NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and separated by electrophoresis on 0.8% (w/v) agarose gels for visualization. The gDNA was sent to the Beijing Genomic Institute sequencing facility (Hong Kong, China) for whole-genome resequencing using the Illumina HiSeq 2000 sequencing system (Illumina Inc., San Diego, CA, USA) with 50x coverage. Briefly, sequencing libraries per gDNA were prepared according to the Solexa sequencing protocols (Illumina Inc., San Diego, CA, USA).
Fig. 5 Population dynamics of different mutant types. Time development of the population fractions for $u = 2 \times 10^{-6}$ and $v/u = 200$. The fractions of the different variants over time in generations are shown on the y-axis, where $F_0$, and $F_m$ are the fractions of cells with $n$ selected mutations for non-mutators and mutators, respectively. Non-mutators (a) contribute almost exclusively to $n = 0$ (red curve) and $n = 1$ (blue curve), while mutators (b) dominate totally in the fractions that carry three or more mutations; in the group with $n = 2$ (green curve), there is some mixing between mutators and non-mutators and samples were dual-indexed and pooled. The paired-end sequence reads have been deposited to the NCBI Sequence Read Archive (SRA) with accession no. SRP133288.

The reads were mapped to the previously sequenced S. typhimurium DA6192 in house reference genome27 using CLC Genomics Workbench version 11 (Qiagen Bioinformatics, Aarhus, Denmark) with default parameters (masking mode = no masking; match score = 1; mismatch cost = 2; cost of insertions and deletions = linear gap cost; insertion cost = 3; deletion cost = 3; length fraction = 0.5; similarity fraction = 0.8; global alignment = no; auto-detect paired distances = yes; non-specific match handling = map randomly). Subsequently, InDels and structural variants were determined following default parameters ($p$-value threshold = 0.0001; maximum number of mismatches = 3; create breakpoints = no; ignore broken pairs = yes; minimum relative consensus coverage = 0.8; minimum quality score = 0; filter variants = no; minimum number of reads = 2; restrict calling to target regions = not set); single-nucleotide polymorphisms were determined following default parameters (ploidy = 2; ignore positions with coverage above = 100,000; restrict calling to target regions = not set; ignore broken pairs = yes; ignore non-specific matches = reads; minimum coverage = 2; minimum count = 2; minimum frequency (%) = 75.0; base quality filter = yes; neighborhood radius = 5; minimum central quality = 20; minimum neighborhood quality = 15; read direction filter = yes; direction frequency (%) = 5.0; relative read direction filter = yes; significance (%) = 1.0; read position filter = no; remove pyro-error variants = no). Sanger sequencing. Mutated regions were PCR amplified with primers binding upstream and downstream of the mutation using DreamTaq DNA polymerase (Thermo Scientific). PCR products were purified and sent to Eurofins MWG Operon (Ebersberg, Germany) for sequencing.

Strain reconstruction. The reconstructed strains were constructed by using Duplication-Insertion Recombineering57 and P22 transduction. A cat-sacB marker (GenBank accession number KM018298) was inserted near each mutation using Lambda-Red recombineering58, generating a marker-hold tandem duplication. The marker and the linked mutation were then moved using P22 transduction into the recipient strains, and the transductants were re-streaked on salt-free LB agar containing 5% sucrose. Only bacteria that lose the cat-sacB marker through recombination between the two copies of the duplicated sequence can grow since expression of sacB is lethal in the presence of sucrose59. The transduced mutations were then confirmed by PCR and sequencing. Gene deletions were constructed by replacing the gene with a cat-sacB cassette, and then removing the cassette with a single-stranded DNA oligo in a second round of recombineering. Primer sequences are listed in Supplementary Table 6. Whole-genome sequencing showed that some of the reconstructed strains carried additional, unintended mutations (Supplementary Table 7). Two of them, yebB (SS4G) and lon (P12syn), were co-transduced from the isolated mutants, while the rest appeared during the reconstruction process.

Growth rate measurements. Growth rates were measured at 37 °C in MH broth using a Bioscreen C Analyzer (Oy Growth Curves Ab Ltd., Helsinki, Finland). Each well was inoculated with a 1000-fold dilution of an overnight culture and measurements were made in quadruplicates. The cultures were grown for 24 h with continuous shaking, and $OD_{595}$ measurements were taken every 4 min. The calculations were based on $OD_{595}$ values between 0.02 and 0.1, where growth was observed to be exponential.
MIC measurements. MIC values were determined by Etest according to the instructions of the manufacturer (bioMérieux, Marcy I’Etoile, France). Etests were performed on MH agar plates incubated for 18–18 h at 37 °C. All MIC values presented are the median values of 2–5 Etests from biological replicates.

Spontaneous mutation rates. The mutation rates of the different strains were estimated with fluctuation assays. From frozen stock, the strains were streaked on MH agar plates and single colonies were used to start overnight cultures in 1 ml MH medium. These cultures were diluted 10^3 in sterile filtered phosphate-buffered saline and 24 independent cultures of each strain were started with an initial inoculum of approximately 1000 cfu in 1 ml MH medium. The cultures were incubated at 37 °C for 20 h to stationary phase, and rifampin-resistant mutants were detected by plating a fraction of each culture on MH agar medium supplemented with 100 mg L^{-1} rifampicin. The volumes plated was 50 µL for non-mutator strains and 5 µL for mutator strains. The population sizes of the cultures were estimated by plating on non-selective MH agar plates. Plates were incubated for 24 h at 37 °C before counting the number of colonies per plate. The estimated mutation rates were calculated with the empirical probability generating function using the web tool be-rates^{8}, with correction for the plating efficiency.

Isolation of total RNA and qRT-PCR. Overnight cultures were diluted 1:100 in 10 ml MH broth and incubated at 37 °C to OD600 = 0.4. To test the effect of excess zinc on adaA expression some cultures were supplemented with 1 mM ZnCl2. 400 µl of bacterial culture was mixed with 800 µl RNAprotect Bacteria Reagent (Qiangen) and total RNA was extracted using the RNaseasy Mini Kit (Qiagen), according to manufacturer’s recommendation. Subsequently, the extracted RNA was treated with DNase Turbo DNA-free kit (Ambion) according to manufacturer’s recommendation and subjected to gel electrophoresis to confirm the integrity of the samples. 500 ng of DNase-treated RNA were reverse transcribed using the High Capacity Reverse Transcription kit (Applied Biosystems), according to the manufacturer’s recommendation. Relative adaA and iraP transcript levels were determined using PerfeCTa SYBR Green SuperMix (Quanta Biosciences) with the Eco Real-Time PCR System (Illumina). The efficiency of each primer pair was determined in a set of six 10-fold dilutions. Expression levels of adaA and iraP were normalized to the reference genes hcaT and cysG. Primer sequences are listed in Supplementary Table 8. Measurements were repeated for at least two biological and three technical replicates each.

Streptomycin uptake assay. To determine the concentration of intracellular dihydrostreptomycin an overnight culture of the strain of interest was diluted 1:200 in 1 ml MH medium. These cultures were diluted 10^3 in sterile filtered phosphate-buffered saline and 24 independent cultures of each strain were started with an initial inoculum of approximately 1000 cfu in 1 ml MH medium. The cultures were incubated at 37 °C for 20 h to stationary phase, and rifampin-resistant mutants were detected by plating a fraction of each culture on MH agar medium supplemented with 100 mg L^{-1} rifampicin. The volumes plated was 50 µL for non-mutator strains and 5 µL for mutator strains. The population sizes of the cultures were estimated by plating on non-selective MH agar plates. Plates were incubated for 24 h at 37 °C before counting the number of colonies per plate. The estimated mutation rates were calculated with the empirical probability generating function using the web tool be-rates^{8}, with correction for the plating efficiency.

Model for mutation accumulation and takeover. We consider a deterministic growth model where back mutations and stochastic effects are neglected. Let $N_n(t)$ be the number of cells with $n$ selected (by $x$ each) mutations at time $t$, and $M_n(t)$ be the corresponding number of mutant cells. They grow with rate $(1+n)s$. Up to five selected mutations can occur in random order each with the basic rate $s$. Mutators are also assumed to occur with rate $u$. When $n$ selected mutations are present, the next one can occur in $5-n$ ways; hence with total rate $u(5-n)(1+n)s/k$. The mutant cells have the basic mutation rate $v$. Then

$$
\frac{dN_n}{dt} = (1+n)sN_n + u(6-n)[1+(n-1)a]KN_{n-1},
$$

$$
\frac{dM_n}{dt} = (1+n)sM_n + v(6-n)[1+(n-1)a]KM_{n-1} + u(1+n)sN_n.
$$

With $N_0$ and $M_1 = 0$ at all times, these equations are valid for $0 \leq n \leq 5$. The initial conditions are:

$$
N_0(0) = N_0,
N_0(0) = 0 \text{ or } N_0(0) = N_0 S_u/s,
N_0(0) = 0; 2 \leq n \leq 5,
M_0(0) = 0; 0 \leq n \leq 5.
$$

Here, $N_0$ is an effective population size. A non-zero initial value for $N_0(0)$ is based on the equilibrium value in a population without antibiotics where a mutation will be counter-selected by $-s$. The solution for the non-mutator part of the population

$$
N_0(t) = N_0 e^{kt},
$$

$$
N_n(t) = N_0 e^{(1+n)kt} \frac{5}{n} \left(\frac{n}{3}\right)^{n-1} \sum_{j=1}^{n-1} (n-j+1) j^{n-j-1} e^{j(1+n)kt}.
$$

The parameter $A = 0$ is for the case with $N_0(0) = 0$ and $A = 1$ corresponds to the case where $N_0(0) = N_0 S_u/s$. The mutators are mostly handled by numerical integration. Let

$$
M_n(t) = e^{(1+n)kt} g_n(t)
$$

Then

$$
g_n(t) = N_0 u t
$$

$$
\frac{dg_n}{dt} = v(6-n)[1+(n-1)a]Ke^{-ut}g_{n-1}(t) + (u(1+n))Ke^{-(1+n)kt}N_n(t); 0 \leq n \leq 5.
$$

The fractions of the different variants at any time can be calculated from

$$
F_n = N_n / \sum_n (N_n + M_n) \text{ and } M_n = M_n / \sum_n (N_n + M_n).
$$

In the calculations, it has been assumed that all selected mutations contribute independently and that each has the same selection coefficient $s = 0.006$, such that five of them give a 3% growth advantage in the presence of the antibiotic. Furthermore, a mutator mutation is assumed to occur with the same rate, $u$, as each of the selected ones. It is straightforward to expand the calculations to include a non-zero initial presence of mutators. However, with an expected fraction on the order of $10^{-6}$, this is found to have a very small effect on the results and is not considered further. The expected initial fraction of single mutants, 5 w/s, may be on the order of $10^{-3}$, which can contribute significantly. Variants with two or more mutations are expected to be present in insignificant fractions initially. Mutators are expected to accumulate deleterious mutations over time contributing to some growth disadvantage for them. Here it is assumed that this effect is small on the time scale considered. Finally, the growth rate $k = \ln(2)$ if time, $t$, is considered in number of generations (doublings). Figure 5 summarizes the time development of the different mutant types.

Without mutators, 1% penetration after 900 generations can be achieved only with a very high mutation rate, $u = 9.5 \times 10^{-4}$, or slightly lower, $u = 7.4 \times 10^{-4}$, if there is a fraction 5 w/s of single mutants present initially. When mutators contribute, this initial condition has little effect and has not been included in the
Calculating epistasis using the weighted Walsh-Hadamard transform. We studied five different genetic loci with either the wild-type or a mutant sequence, making a total of 25 = 32 genotypes, so the collection of all 32 reconstructed mutants with resistance phenotypes (Fig. 1 and Table 2) represents a combinatorially complete dataset for the five mutations. The fitness landscape can be seen as a high-dimensional space where different orders of epistatic (non-additive) interactions can be calculated through spectral analysis using transformation theory, similarly to how a Fourier transform can be used to decompose a signal in the time domain into different components in the frequency domain. The higher-order epistatic effects of the different resistance mutations were calculated through Walsh–Hadamard transform decomposition of the fitness landscape of streptomycin resistance. Since this is a linear epistasis model while antibiotic resistance increases in a multiplicative way, the phenotypic resistance values were linearized as log₂(MIC).

The epistatic effects were calculated in two different ways: as the global epistatic effect of each mutation (background-averaged epistasis, corresponding to a Fourier expansion), and as the epistatic effect of each mutation with the wild-type sequence (corresponding to a Taylor expansion), similarly to how a Fourier transform can be used to decompose a signal in the time domain into different components in the frequency domain. The higher-order epistatic effects of the different resistance mutations were calculated through Walsh–Hadamard transform decomposition of the fitness landscape of streptomycin resistance. Since this is a linear epistasis model while antibiotic resistance increases in a multiplicative way, the phenotypic resistance values were linearized as log₂(MIC).

We first calculated the global, background-averaged epistasis ε; the epistatic effects of each mutation as an average over all possible sequence variants. In this case, we can define

\[ \Omega_{sp,global} = VH, \]

where V is a weighting matrix to normalize for the different numbers of terms for epistasis of different orders, recursively defined as

\[ V_{n+1} = \left( \begin{array}{cc} V_n & 0 \\ 0 & V_n \end{array} \right) \text{with } V_0 = 1 \]

and the matrix H corresponds to the Walsh–Hadamard transform generated by the recursive definition

\[ H_{n+1} = \left( \begin{array}{cc} H_n & H_n \\ H_n & -H_n \end{array} \right) \text{with } H_0 = 1. \]

The vector of background-averaged epistatic terms \( \mathbf{T} \) can then be calculated by

\[ \mathbf{T} = VH\mathbf{\tilde{y}}. \]

To calculate the vector of epistatic terms \( \mathbf{T} \) relative to the wild-type sequence, we instead use

\[ \Omega_{sp,\mathbf{x}^T} = VX^T H. \]

where \( X^T \) only includes the terms associated with the wild type, and the matrix X can be defined as

\[ X_{n+1} = \left( \begin{array}{cc} X_n & 0 \\ X_n & X_n \end{array} \right) \text{with } X_0 = 1, \]

which gives us

\[ \mathbf{T} = VX^T H\mathbf{\tilde{y}}. \]

The calculations were performed using Python 3.6 with the NumPy library.

**Code availability.** Python scripts are available from the corresponding author upon request.
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Author contributions
D.I.A., E.W.Y., and M.K. designed the experiments; E.W.Y., M.K., K.H., and S.K. performed the experiments. E.W.Y., M.K., O.G.B., D.I.A., and K.H. analyzed the data. E.W.Y., M.K., and D.I.A. wrote the manuscript with contributions from all authors.

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