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Review Article

Integrating bacterial molecular genetics with chemical biology for renewed antibacterial drug discovery

Susannah L. Parkhill[1,2] **and Eachan O. Johnson**[1,2,3,4]

[1]Systems Chemical Biology of Infection and Resistance Laboratory, The Francis Crick Institute, London, U.K.; [2]Faculty of Life Sciences, University College London, London, U.K.; [3]Department of Chemistry, Imperial College, London, U.K.; [4]Department of Chemistry, King's College London, London, U.K.

Correspondence: Eachan O. Johnson (eachan.johnson@crick.ac.uk)

The application of dyes to understanding the aetiology of infection inspired antimicrobial chemotherapy and the first wave of antibacterial drugs. The second wave of antibacterial drug discovery was driven by rapid discovery of natural products, now making up 69% of current antibacterial drugs. But now with the most prevalent natural products already discovered, ~10^7 new soil-dwelling bacterial species must be screened to discover one new class of natural product. Therefore, instead of a third wave of antibacterial drug discovery, there is now a discovery bottleneck. Unlike natural products which are curated by billions of years of microbial antagonism, the vast synthetic chemical space still requires artificial curation through the therapeutics science of antibacterial drugs — a systematic understanding of how small molecules interact with bacterial physiology, effect desired phenotypes, and benefit the host. Bacterial molecular genetics can elucidate pathogen biology relevant to therapeutics development, but it can also be applied directly to understanding mechanisms and liabilities of new chemical agents with new mechanisms of action. Therefore, the next phase of antibacterial drug discovery could be enabled by integrating chemical expertise with systematic dissection of bacterial infection biology. Facing the ambitious endeavour to find new molecules from nature or new-to-nature which cure bacterial infections, the capabilities furnished by modern chemical biology and molecular genetics can be applied to prospecting for chemical modulators of new targets which circumvent prevalent resistance mechanisms.

Introduction

Antibacterial drugs form the backbone of modern medicine by not only curing infections but also supporting essential interventions including organ transplantation and cancer treatment. Alarmingly, in 2019, an estimated 4.95 million deaths worldwide were associated with antimicrobial resistance [1,2]. Compounding this issue, the half-life of an antibacterial agent in the clinic before detection of resistance is ~15 years (Figure 1A,B), requiring constant discovery to sustain an inventory of clinically useful antibacterial drugs which circumvent prevalent resistance mechanisms. In the last 30 years, the rate of discovery of these drugs has fallen behind the emergence of new resistance, such that there is now resistance to every clinically approved antibiotic (Figure 1C). As a result, antibacterial drugs are a perishable common good requiring a strategy for sustainable discovery.

The present torpor emerges from peculiarities of the history and practice of antibacterial drug discovery. First, the failure of target-based discovery [4,5] has made the target-agnostic phenotypic approaches necessary, resulting in increasing re-discovery of easily drugged targets. Second, a focus on natural products has led to an underdeveloped antibacterial therapeutics science which would otherwise guide the discovery and development of new synthetic antibacterial drugs.

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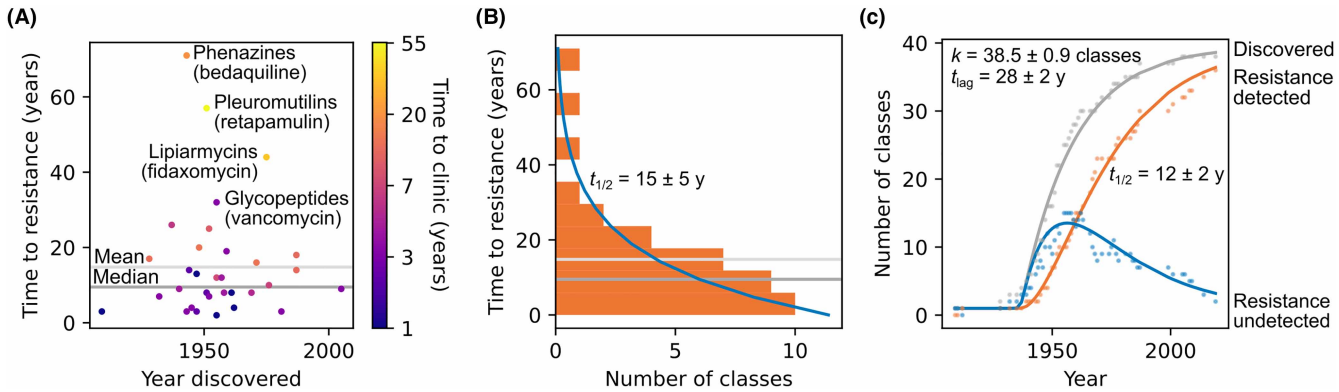

Figure 1. The rate of antibacterial drug discovery has reached a plateau, while the rate of emergence of new resistance is constant.
(**A**) The time between discovery and resistance detection compared with the discovery year for classes of antibacterial drugs. Colour indicates the time between discovery and clinical use. Outliers in time to resistance (labelled with a canonical example in parentheses) are characterised by a longer gap between discovery and clinical use. (**B**) The distribution of time between discovery and resistance for classes of antibacterial drugs follows an exponential distribution (Poisson maximum likelihood fit shown by blue line), with a half-life of ∼15 years (95% confidence interval: 10–20 years). (**C**) Poisson maximum likelihood best fits for the dynamics of antibacterial drug discovery. The number of classes with resistance not-yet-detected (blue line) as a function of time was modelled as the difference between the number of discovered classes, D (grey line), and the number of classes with detected resistance, R (orange line). R was modelled as exponential growth with rate $1/t_{1/2}$. D was modelled as the coupon collector problem when sampling from k antibacterial drug classes at a constant rate n after a ∼28 year lag phase, t_{lag}. Data were obtained from Stennett etal. [3].

Examining the early history of antibacterial drugs reveals unexplored paths to renewed discovery which might guide the application of the powerful genome-scale genetic and chemical tools now at our disposal. In an early example of integrative chemical biology, in 1882, the dye methylene blue revealed to Robert Koch the bacterium *Mycobacterium tuberculosis* as the cause of tuberculosis, inspiring the development of his eponymous postulates. This fundamental biological discovery using chemistry inspired potential cures: contemporaneously with Koch, Paul Ehrlich's selective staining of specific mammalian cells, such as mast cells, led to concept of the magic bullet, a chemical which partitions into disease-causing cells and harms them, leaving host or normal cells intact. Soon after, trypan red (named for its activity against trypanosomes) was discovered as the first antimicrobial magic bullet in 1904. In 1909, Ehrlich himself identified, with the assistance of Sahachiro Hata, the first commercial antibacterial agent, arsphenamine, as a cure for syphilis. Inspired by this progress, dye companies pursued systematic derivatisation and screening of their inventories and reaction side-products against microbes [6,7]. This effort led to the first wave of discovery of effective antibacterial drugs starting with the prodrug, prontosil by Gerhard Domagk in 1932 and its active metabolite, sulfanilamide, in 1935.

The second wave of antibacterial drug discovery followed the isolation of penicillin from the *Penicillium chrysogenum* fungus in 1940 [8],demonstrating for the first time that natural products could be harnessed not only as narcotics (such as opium and digitoxin) but also as life-saving medicines. This conceptual revolution provided benefits beyond curing infectious disease, including cancer treatment (anthracyclines, etoposide, vinca alkaloids) and prevention of organ transplant rejection (rapamycin, cyclosporin). For most bacterial infections, natural product screening and development of semi-synthetic analogues overtook synthetic chemistry approaches in a golden era of antibiotic discovery during the mid-20th century [7,9,10]. Now, 69% of antibacterial drugs are natural products derived from soil microbes, in contrast with the entire pharmacopoeia which comprises 38% natural products [11].

Notable exceptions are the antituberculosis drugs, most of which are synthetics from early screening of dye manufacturing by-products, despite the first effective tuberculosis chemotherapy being streptomycin, a natural product derived from the bacterium *Streptomyces griseus*. Streptomycin, discovered by Albert Schatz, Elizabeth Bugie, and Selman Waksman in 1944, caused a high incidence of allergies and relatively fast resistance acquisition by *M. tuberculosis* (factors which led the death of the author George Orwell in 1950). Therefore, it was eventually combined with synthetic *para*-aminosalicylic acid as the standard of care following a landmark clinical trial in 1950 [12]. Since then, streptomycin is no longer routinely used, with rifampicin the only natural product among first-line antituberculosis drugs.

The post-genomic age of the late 20th century promised a third wave of antibacterial drug discovery. It was thought that molecular biology, which provided the ability to identify new targets unique to pathogens, would yield numerous magic bullets through rational design [13]. However, high-throughput biochemical screening against known or novel targets *in vitro* over the last two decades have resulted in no clinical antibacterial candidates, with retrospective analyses noting an apparent bias towards tight-binding compounds with high lipophilicity, and subsequent difficulties in developing whole-cell activity [4,14]. This failure of target-based screening taught the harsh lesson that a drug is more than an avid binder of a validated target.

As a result, target-agnostic phenotypic screening for antibacterial candidates has returned to prominence along with an appreciation for natural products. However, discovery is stymied by the coupon collector problem, where drawing from the same pool of natural products at first yields a rapid increase in new discoveries — the golden age — followed by repeated re-discovery (Figure 1C). This phenomenon became so acute that by 1958 commercial natural product mining efforts invested in dereplication platforms to weed out rediscoveries early in their pipeline [15], but now with the most prevalent agents already discovered, to yield one new antibacterial natural product requires screening around 10^7 new bacterial species [16].

Therefore, instead of a third wave of antibacterial drug discovery, there is now a discovery bottleneck (Figure 1C). Since the staining of *M. tuberculosis* with methylene blue, our understanding of bacterial biology and infection processes has increased dramatically, but in the last 10 years, all 19 approved new antibacterial drugs belonged to existing classes, except for antituberculosis drugs pretomanid (2019) and delamanid (2014) [17–20]. Of seven antibiotics in new drug application or Phase III clinical trials at the end of 2022, only gepotidacin (for urinary tract infections and gonorrhoea) and zoliflodacin (for gonorrhoea) are novel pharmacophores and only epetraborole (for *Mycobacterium avium* infection) has a new mechanism of action. None are found in nature, indicating the potential of synthetics to furnish new drugs with new mechanisms of action.

The proportion of synthetic compounds (from 44% to 61%) and of novel pharmacophores or mechanisms of action (28% to 65%) has increased in all phases of clinical trials from 2011 to 2022, indicating a promising shift away from me-too drugs and natural products to which resistance likely already exists in the environment [17,21]. However, unlike natural products which are selected and enriched by billions of years of microbial antagonism [22], the vast synthetic chemical space requires artificial curation, which can be assisted by integrated chemical and molecular genetic approaches to discover new compounds active against pathogenic bacteria.

Integrating chemistry with genetics for renewed discovery

The conventional role of molecular genetics in drug discovery has been to implicate gene products in disease, thus prioritising them for target-based compound screening. However, unlike many drugs such as ivacaftor (restoring CFTR activity) for cystic fibrosis, imatinib (inhibiting tyrosine kinase) for leukaemia, and saquinavir (protease inhibitor) for HIV, the targets of antibacterial drugs were rarely known before clinical use. Indeed, the mechanism is still debated in several cases, including, for example, pyrazinamide for tuberculosis [23,24]. This ignorance does not preclude clinical value, but rather indicates complex, multi-target activities which drive efficacy and raise the barrier to resistance. As a result, most approved antibacterial drugs were empirically identified as killing a pathogen or curing a host [5], with target-based efforts to discover new antibacterial drugs almost universally failing [4].

Instead of simply prioritising individual gene product targets for compound screening, target-agnostic phenotypic approaches yield empirically effective candidates without prejudice towards any particular target. As chemical matter discovered this way progresses through the pre-clinical pipeline, it is advantageous to elucidate mechanisms of action and resultant implications for resistance and drug combinations. Molecular genetics can address this need, and help fill remaining knowledge gaps in the field of antibacterial drug discovery (Figure 2A). For example, the emerging discipline of chemical genomics might enable efficient target dereplication to prioritise chemical matter which could circumvent prevalent resistance mechanisms. Using genetics to build better understanding of how cells modify or exclude chemical compounds would enable prioritisation or design of compounds which are not easily inactivated or excluded. Additionally, once the target of an antibacterial candidate discovered using target-agnostic methods is known, the network of gene–gene interactions would indicate productive combination regimens that increase efficacy and lower the incidence of resistance (Figure 2B).

However, even with the most detailed understanding of pathogen biology, new antibacterial drugs will not be discovered without tools to systematically understand how this biology reacts to perturbation by new natural

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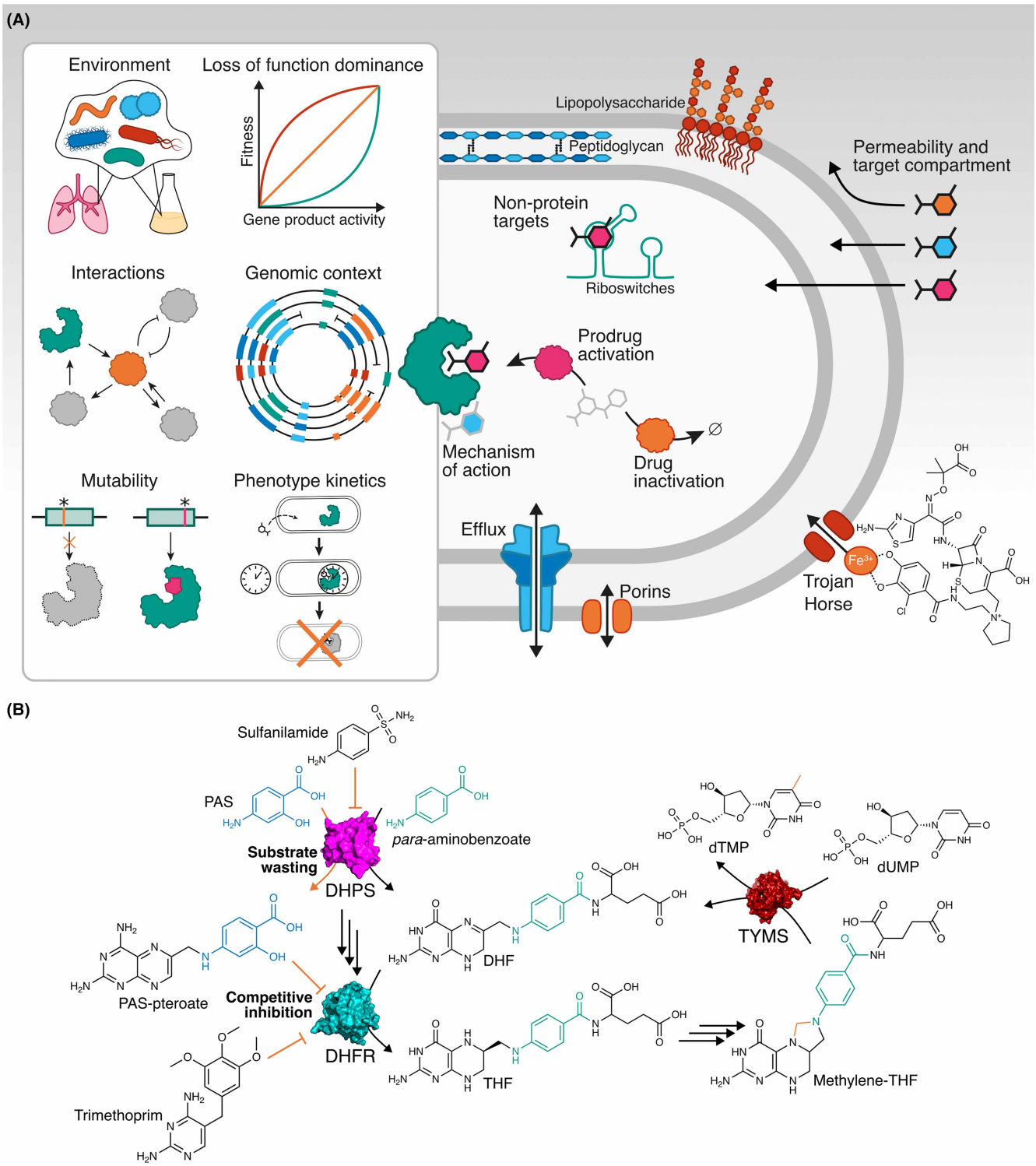

Figure 2. Biological approaches integrated with chemical understanding are essential for antibacterial drug discovery. Part 1 of 2

(**A**) Putative targets of hit compounds can be prioritised using genetic approaches (left panel) to assess their likely efficacy and propensity for resistance. The fate and efficacy of bioactive compounds is not only determined by target, but also by chemical properties which influence uptake, mechanism of action, and, again, propensity for resistance. (**B**) The folate biosynthesis pathway is an exemplar of multiple mechanisms of action including competitive inhibition (trimethoprim) and substrate wasting (PAS), as well as how knowledge of biological networks can explain synergy (trimethoprim and sulfonamides) and resistance liabilities (TYMS loss of function renders DHFR non-essential). Atoms conserved in downstream

Figure 2. Biological approaches integrated with chemical understanding are essential for antibacterial drug discovery. Part 2 of 2
metabolites are indicated in colours. DHFR, dihydrofolate reductase; DHF, dihydrofolate; DHPS, dihydropteroate synthase; PAS, *para*-aminosalicylic acid; THF, tetrahydrofolate; TYMS, thymidylate synthase.

products and synthetic chemical agents. Similarly, new drugs will not enter the clinic without antibacterial medicinal chemistry for permeability, low toxicity, and pharmacokinetic and pharmacodynamic properties (Figure 2A). Therefore, the next phase of antibacterial drug discovery will be enabled by integrating this chemical expertise with systematic dissection of bacterial infection biology.

Genetics across scales: informing antibacterial drug development

The sustainable efficacy of an antibacterial drug depends on how its effect on gene product activity changes infection outcome, and how likely it is that resistance will evolve either to its mechanism of action or to its chemistry. Genetic perturbations can roughly simulate the effect of a non-competitive drug, but their pertinence in drug discovery is in understanding genes' roles in broader cellular, evolutionary, and chemical biology of infection. Within phenotypic screening paradigms, molecular genetics can also aid target deconvolution for screening hit compounds, enabling subsequent prioritisation on target attractiveness. Multi-scale molecular genetics can assess several factors influencing this attractiveness, including dominance of its loss of function, dependence of this dominance on genomic context, kinetics of phenotype, and its mutability (Figure 2A).

Genes: loss-of-function dominance

Conventional antibacterial drugs inhibit one or more gene products whose function is essential. Since the concentration of drugs in a host's tissues fluctuate between doses, ideal targets are those upon which a bacterial phenotype, such as growth rate, is exquisitely dependent, so that modest loss of function causes catastrophic loss of fitness. Recent genetic tools provide a means to assess thousands of genes in parallel for this loss-of-function dominance (Figure 3A).

Gene deletion has for decades been used to understand fitness dependence on gene dosage in model polyploid species like *Saccharomyces cerevisiae*, but this method cannot be trivially extended to essential genes in haploid bacteria, since deletion is lethal [25]. Generating clean gene deletion mutants quickly becomes laborious at the genome scale, but their utility drove investment in deletion libraries of all non-essential genes for

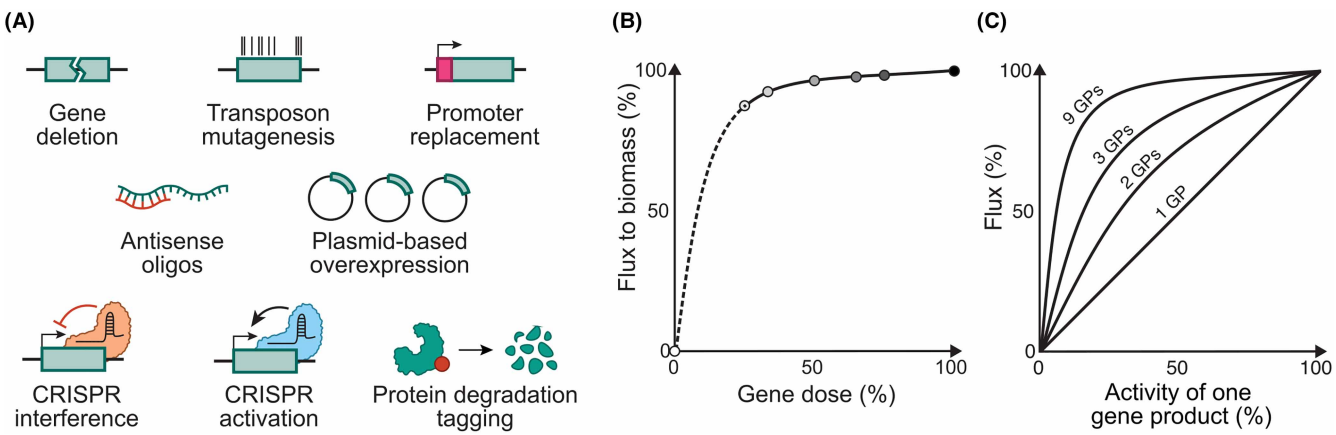

Figure 3. Genetic tools to understand the function of specific drug targets as well as their context in wider biological networks.
(**A**) Schematic of methods to determine gene essentiality, function, and loss-of-function dominance. (**B**) Growth of *S. cerevisiae* mutants at the *ad-2* locus, involved in purine biosynthesis. Black circle indicates wild-type activity, grey circles indicate triploid and tetraploid strains with null and wild-type allele combinations. (**C**) Effect on flux of decreasing activity of one gene product (GP), with increasing pathway size (1, 2, 3, and 9 gene-products). Adapted from Kacser and Burns [25].

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model organisms (*Escherichia coli*, *Bacillus subtilis*) and some pathogens (*Acinetobacter*, *Salmonella* spp.) [26–29]. Unfortunately, such libraries do not exist for many important pathogens including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *M. tuberculosis* because genetic manipulation in these species is inefficient or only recently implemented [30,31]. The effect of gene deletion on fitness can also be defined through disruption of open reading frames by random transposon insertion, usually resulting in inactivation of the target, and has been used to create single mutant libraries in less genetically tractable species such as *P. aeruginosa*, *K. pneumoniae*, *M. tuberculosis*, *Neisseria meningitidis*, *Vibrio cholerae*, and *Francisella novicida* [32–43]. Comparison of essentiality analysis from deletion and transposon insertion libraries showed overlap between each method, but transposon libraries revealed greater resolution on essential regions of genes, polarity effects, and other genomic features like protein secondary structure. For example, a single insertion in the essential region of nucleotide exchange factor *grpE* demarcates a flexible region between two essential α-helices involved in DnaK binding [44,45]. Like other high-throughput methods, transposon-based methods are limited by the polar effect arising from disrupting operon structure, sequence insertion bias, insufficient saturation, and *trans*-complementation during pooled growth [45–47].

Given that essential genes tend to be highly conserved, antibacterial drugs targeting essential genes often possess broad-spectrum activity [48]. However, the dichotomy of essentiality does not capture the continuous response of phenotype to gene dosage. For haploid organisms (including most bacterial pathogens), this continuous response can now be measured using more recent molecular techniques interfering with transcription, translation, and post-translational processes.

Ideal inhibitors would target an essential gene whose modest loss-of-function would result in a large fitness decrease. Loss-of-function dominance (also termed vulnerability), is the quantitative relationship between gene-product activity and organism fitness, first studied by reducing gene dosage in polyploid organisms (Figure 3B, C). In principle, vulnerable genes make good drug targets because small molecules might not always realise total inhibition of gene product activity, and invulnerable essential genes can tolerate such partial inhibition without fitness impacts [49,50]. Additionally, invulnerability implies that most mutant alleles are recessive in fitness, allowing exploration of genetic space — including evolution of target-based drug resistance — without catastrophic fitness loss. After target deconvolution for hits from phenotypic screening, an important property to inform prioritisation of candidates is the dominance of their target's loss of function.

To that end, measurement of phenotypes as a function of gene transcription levels was enabled for the first time by controlling the transcription of single genes of interest through expression from a plasmid or fusion of synthetic promoters to the endogenous open reading frame [51–57]. Enabling genome-scale application of this approach, CRISPR interference (CRISPRi) sterically blocks native transcription at a programmed locus using a catalytically inactive Cas9 nuclease (dCas9) directed by a guide RNA (gRNA) protospacer provided in *trans* [58,59]. The extent of CRISPRi can be modulated through titration of dCas9 or gRNA expression, mismatches between the protospacer and target sequence, or protospacer adjacent motif sequences [50,60,61]. Although mitigation strategies are emerging, CRISPRi can, like transposon mutagenesis, exert unintended polar effects on polycistronic operons and off-target loci [62–68]. Despite these drawbacks, CRISPRi has been applied extensively in *E. coli*, *B. subtilis*, and *M. tuberculosis*, where pooled and arrayed libraries targeting each gene or tiled across the genome have been used to survey vulnerability and function, including comparison between strains and conditions [63,68–72]. Measuring phenotypes beyond simple fitness, screens have combined CRISPRi with imaging, animal infection models, and study of gene–environment interactions [70,73–75].

CRISPR activation, where CRISPRi machinery is paired with a transcriptional activator to recruit RNA polymerase and up-regulate transcription, is well-established in eukaryotes but equivalent prokaryotic tools are still under development [58,76–80]. Refinement of this technique will allow investigation of targets amenable to non-inhibitory chemical modulation, currently only achievable through lower-throughput promoter replacement tools, such as the DegP and ClpP proteases which are activated by antibiotic acyldepsipeptides (ADEPs) [81]. However, transcriptional modulation only provides a partial model of chemical interaction with gene products, and can be confounded by translation rate and protein stability [82].

Translation can be manipulated using antisense oligos to bind a gene transcript resulting in steric hindrance of translation or degradation of the antisense-RNA complex [83,84]. Shotgun antisense RNA (asRNA) cloning enabled comprehensive genome-wide screening, but identified essential genes can be variable: two separate asRNA assays in *Staphylococcus aureus* identified 150 and 658 essential genes, and an asRNA screen in *E. coli* only identified one-quarter of essential genes previously identified by gene deletion [26,85–88]. This discrepancy may result from incomplete asRNA silencing or leaky asRNA expression preventing generation of

knockdown strains targeting essential genes expressed at a low level. Nevertheless, antisense oligos are well-suited to study of riboswitches as drug targets, and have themselves been developed as antibacterial agents [89,90]. However, conventional antibacterial drugs act post-translationally; therefore, genetic tools acting similarly give important insights into the biology of drug targets.

The first techniques for tuneable post-translational proteolysis applied carboxy-terminal SsrA-like tags to a coding sequence of interest and used adaptor SsrB to regulate degradation of the tagged fusion by caseinolytic protease Clp [91–93]. Later developments involved chemically induced reassembly of split adaptors, regulation through tag cleavage, or orthogonal tags and heterologous machinery [94–96], while orthologous methods for protein degradation include BacPROTACs [97]. Protein degradation is a fast-onset perturbation and enables investigation of long-lived proteins whose copy number is robust to transcriptional modulation. However, it does not recapitulate conventional chemical inhibition, where the protein remains intact and can perform other functions such as scaffolding, alternative reactions, or substrate wasting. For example, fluoroquinolones cause DNA gyrase to generate breaks in DNA, which does not happen on transcriptional or post-translational depletion of the enzyme [98,99]. Additionally, not all targets are susceptible to proteolysis, and, as shown in studies on *E. coli* and *M. tuberculosis*, not all genes are amenable to tagging, potentially due to interference with functionally essential multimerisation [96,100].

Importantly, gene dose–phenotype response is distinct from any compound dose–response, partly because the response curve will depend on whether a compound acts competitively or allosterically, partly because most chemical inhibitors act post-translationally (thus subject to different compensatory mechanisms, such as metabolite feedback inhibition), and partly because compound affinity and partitioning will determine the degree of gene product inhibition and thus its phenotypic consequences. A genetically vulnerable gene whose product is undruggable is chemically invulnerable, whereas a genetically less vulnerable gene for which a high affinity inhibitor exists is chemically vulnerable. This concept is demonstrated by the handful of bacterial genes that have known chemical inhibitors, compared with the hundreds of vulnerable genes that have been reported. Effective drugs are more than their targets; therefore, integrated biological and chemical approaches are indispensable for developing new antibacterial drugs.

Nevertheless, loss-of-function dominance is an attractive property of potential drug targets, emergent from a gene product's kinetic linkage to other genes through shared metabolites and protein–protein interactions. Loss-of-function dominance is, therefore, dependent on the gene product's context within metabolic and regulatory networks [25], resulting in a non-linear relationship between gene product activity and fitness (Figure 3B). Because vulnerability depends on local interactions in biological networks, systematic identification of gene–gene interactions would deepen our understanding of vulnerability as well as its contribution to the evolution of resistance (Figure 3C).

Interactions: metabolic control and synergy

To quantitatively elucidate the kinetic linkage between two gene products, they must be modulated in combination [101]. In *S. cerevisiae*, systematic study of genetic interactions yielded information on functional and regulatory relationships, enabling functional prediction for uncharacterised genes [102,103]. Quantifying genome-wide epistasis in bacteria will reveal genes that are metabolically coupled and therefore constrained to co-adapt. This knowledge could lead to prediction of available evolutionary routes in response to antimicrobials. Additionally, genetic interactions can indicate synthetic lethality to be exploited or synthetic rescue to be avoided in drug combinations.

In haploid bacteria, although double deletion mutants can be constructed to measure a single interaction between two genes, this approach is limited to pairs of non-essential genes, and yields only binary rather than quantitative data on gene–gene interactions. Current low-throughput methods involve bacterial conjugation, which is hampered by false positive interactions arising from decreased recombination efficiency between genes within 60 kb of each other, and arrayed plate growth, limiting throughput compared with pooled methods [104,105]. Alternatively, transposon insertion sequencing can be performed in a mutant background, but this has not yet been applied to all gene–gene pairs [32].

Barcoded shotgun and recombineering strategies for overexpression has dramatically increased throughput, affording pairwise and higher-order combinatorial overexpression of transcription factors to identify network interactions that potentiate antibiotics [106–109]. CRISPRi made gene–gene titration accessible through combination of CRISPRi libraries and gene deletion collections, or use of multiple co-ordinately controlled gRNAs to quantify synergistic and synthetically lethal interactions [59,110–114]. Recently, mismatch CRISPRi has been

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used in *E. coli* to generate independently titrated pairwise gene knockdowns for 19 genes, but genome-wide quantification of genetic epistasis represents a combinatorial challenge that has not yet been resolved for any organism [115].

The fitness of mutants in the presence of a chemical compound, i.e. chemical–genetic interactions, can also be used to evaluate antibacterial drug targets [98]. In diploid organisms, heterozygous gene deletion produces strains hypersensitised to drugs that act on the corresponding gene product. Therefore, the combination of gene deletion and chemical screening allows identification of compound mechanisms of action for target dereplication [116]. Combination of deletion and transposon insertion libraries with antibiotics and other chemical stressors elucidated non-essential gene function, conditional essentiality, and chemical–genetic interactions, whilst also identifying antibiotic resistance determinants and secondary targets whose inhibition could potentiate existing antibiotics in combination therapy [117–125]. For example, hypersensitive asRNA knockdown strains were used in natural product screens to identify inhibitors of fatty acid synthesis and ribosomal machinery [126–128]. Similarly, the PROSPECT assay combines sensitised strains produced through degradation tagging with chemical libraries, enabling systematic screening for new compounds with whole-cell activity [129].

CRISPRi can also be combined with chemical screening, allowing prediction of chemical mechanisms of action and synergistic targets, and identification of intrinsic drug resistance mechanisms [71,130,131]. Metabolic profiling of CRISPRi and chemical perturbations identified shared signatures between chemical inhibition and target knockdown. However, some targets present exceptions where genetic knockdown does not phenocopy or causes opposite effects to antibiotic action, such as the effect of fluoroquinolones on *gyrA* [130], further emphasising the risks of overinterpreting transcriptional inhibition as chemical modulation.

Chemical–genetic interactions can also reveal resistance liabilities of antibacterial drugs. For example, in the PROSPECT study [129], the MshC knockdown strain of *M. tuberculosis* was resistant to isoniazid, highlighting mycothiol biosynthesis loss of function as a resistance pathway [132]. The same work showed the ThyA (thymidylate synthase) knockdown to be resistant to folate biosynthesis inhibitors, a phenomenon recapitulated in the clinic with *thyA* deletion conferring resistance to *para*-aminosalicylic acid, a dihydropteroate synthase inhibitor (Figure 2B) [133]. However, in evaluating resistance liabilities of antibacterial drug targets, disruption of entire gene products can only model resistance conferred by loss of function, whereas prevalent examples of resistance are conferred by acquisition of new alleles, or abolition of target binding through mutation.

Mutability: acquisition of resistance

The driver of our constant search for new antibacterial drugs is resistance, which emerges from interactions between a drug's chemistry and multi-scale pathogen biology from genes through communities and host–pathogen interactions. Genes encoding resistance to β-lactams, tetracyclines, and glycopeptides have been identified in environmental samples dating back 30 000 years, and phylogenetic analyses indicate antibiotic synthesis and resistance genes coexisted hundreds of millions of years ago [134–137]. Evolution of antibiotic synthesis genes and resistance mechanisms was likely driven by early competition [138–141]. Such resistance mechanisms can be generic (reduced influx, efflux), target specific (target modification, variation, or protection, metabolic bypass), or class-specific (compound modification, prodrug activation, efflux), and mobilised between species though horizontal gene transfer. Since many different forms of resistance to natural product antibiotics are widespread in non-pathogenic environmental bacteria [142,143], it is increasingly important to proactively assess resistance liabilities of both the chemical class and the target of antibiotics in development.

Whereas resistance to natural products commonly occurs through gain of function resistance transmitted by mobile genetic elements, including target protection and drug inactivation, it can also occur through target mutation or even polymorphisms that are apparently unrelated to the mechanism. For example, mutation of RNA polymerase subunits in *Neisseria gonorrhoeae* confer resistance to cefalosporins which inhibit cell wall biosynthesis [144]. Similarly, resistance to synthetic antibiotics often occurs through chromosomal mutation of their target or prodrug activating enzymes [145–147]. Therefore, when prioritising new small molecules for their potential as antibacterial drugs, it is important to consider the resistance liabilities conferred by both their chemistry and their targets. New resistance-conferring alleles, such as inactivating enzymes [148] or efflux pumps [149], usually act on the chemistry of a drug and originate as immunity factors or mutated biosynthesis genes in the producer of a given natural product antibiotic [150]. Alternatively, target mutations reduce the binding of a compound while retaining essential target function, as with rifampicin, which inhibits RNA

polymerase. For these cases, molecular genetics enables quantification of target mutability and plasticity of compensatory pathways which would allow regimen design to anticipate resistance.

The propensity for a target to acquire resistance while retaining enough essential activity to maintain viability depends on three influences. First, evolution has tuned genomic stability by balancing the opposing forces of DNA repair fidelity and DNA damage, both by endogenous mechanisms (such as replication–transcription conflicts [151]) and by exogenous insults (such as host reactive oxygen species, ultraviolet light, and drugs like mitomycin). These forces depend on local nucleotide sequence, structure, and gene expression; therefore, a target's mutability depends partly on its encoding gene's accessibility to mutagenic mechanisms. Second, the dominance of the gene's loss of function determines the tipping point of the resistance-fitness trade-off. Third, the functional fragility of a target will determine which regions of genetic sequence space are under purifying selection. If a protein target's activity or function can tolerate few mutations, then it is less likely that one of those mutations would confer resistance to a drug. For example, the small molecule BRD-8000.3 inhibits *M. tuberculosis* EfpA, an essential efflux pump, but the handful of target-based resistance mutations also cause severe fitness defects, and multiple mutations are empirically not tolerated [152].

The fragility of gene function is also influenced by context within metabolic, regulatory, and protein–protein interaction networks, as demonstrated by the influence of a prevalent carbonic anhydratase mutant of *N. gonorrhoeae*, which causes CO_2-dependence but compensates fitness loss from DNA gyrase mutations, which confer fluoroquinolone resistance [153]. On the one hand, biological networks define evolutionary constraints with respect to metabolically coupled genes (e.g. dihydrofolate reductase and thymidylate synthase), directly interacting gene products (e.g. two-component signalling systems), or functionally gratuitous dimers with hydrophobic interfaces (e.g. steroid hormone receptors in eukaryotes) [154–156]. These constraints ensure that core metabolism is conserved across the domains of life because it is limited by thermodynamics, stoichiometry, and requirements for certain reaction products [157]. On the other hand, network complexity ensures that no single gene product is responsible for a rate-limiting step, but rather all gene products exert differing degrees of metabolic control on a pathway. This metabolic control emerges from the kinetics of molecular interactions, which also determine the clinical efficacy of antibiotic drugs.

Time: phenotypic kinetics

Typical serum concentrations of a drug during antibacterial chemotherapy display a pulsatile time-dependence; therefore, an ideal drug target will be vulnerable to lower drug concentrations and have long-lasting phenotypic effects even after brief treatment. The post-antibiotic effect, where bacterial growth is suppressed after removal of drug, depends on both antibiotic concentration and exposure time, with this lag being attributed either to dissociation of antibiotic from the target (a chemical property) or to resynthesis of the target (a biological property) [158–161]. Similarly, the duration of chemical stress dictates the bacterial cell response to such stress [162]. Clearly, duration of drug exposure is a key consideration when defining therapeutic dosage and schedule. It is especially important for pathogens that occupy niches difficult for antibiotics to penetrate, such as lesions formed during *M. tuberculosis* infection [163].

To explore the sensitivity of targets to inhibition duration, one effort measured depletion sensitivity, the speed of growth inhibition after gene inactivation through transposon mutagenesis [164]. The resulting metric was used to rank essential processes by their importance for replication. Due to the inherent differences between chemical inhibition and genetic inhibition, temporal vulnerability to an inhibitor cannot be interpreted from depletion sensitivity, because depletion sensitivity will be affected by transcript dose, protein levels and turnover, which are unlikely to factor into chemical inhibition of a gene product in the same way.

So far, phenotypic kinetics has only been studied in single representative strains from a given species. However, since many bacterial species have large open pangenomes and high variation in gene content within a species, strain background will influence most molecular genetic characterisations relating to the targets of antibacterial drugs [165].

Genomic context: spectrum of activity

Within *Enterobacteriaceae*, wide variation in essentiality has been identified between strains of the same species, and up to one-third of essential genes are non-essential in other species within the same genera [63,166]. Essential genes have recently been reclassified to describe this flexibility: universal (present and essential across the pangenome), core strain-specific (present in all strains, essential in some), and accessory essential

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(essential when present) [167]. Importantly, core strain-specific and accessory essential genes can become non-essential with genetic background changes.

This effect propagates to chemical inhibition. Studies of MetS inhibitors in *Streptococcus pneumoniae* demonstrated highly heterogenous activity between strains, with 20% of strains resistant to the inhibitor due to a second redundant MetS that is widespread but not universal [168]. Therefore, genomic context is critically important when examining the properties of antibacterial drug targets, but recent work indicated that this issue is more tractable than might be thought, since analysis of only four strains was sufficient to identify core essential genes shared across strains and relevant growth conditions [45]. Nevertheless, this threshold will likely differ by species, and even single polymorphisms can affect secondary phenotypes like the ability to evolve drug resistance [153]. Transposon mutant libraries can easily be made in multiple strains of a species (including clinical isolates) to allow understanding of variation in essentiality across the pangenome, but they can also be easily subjected to a range of environments, including infection [45,169,170]. If not studied under relevant conditions, empirically high value targets identified by such screens can be misleading.

Conditions: environmental and host interactions

Cautionary tales abound of antibacterial candidates targeting gene products which are essential in axenic culture but dispensable during infection. In one example, pyrimidine–imidazole inhibitors were developed against for *M. tuberculosis* with high *in vitro* potency but no *in vivo* activity because the target, glycerol metabolism, was irrelevant during infection [171–173]. This effect extends to the fundamental study of gene essentiality, where screening of 24 conditions allowed identification of novel condition-specific essential genes in *Staphylococcus epidermidis*, as well as those vital for adaptability to environmental pressures [66]. In addition, a study of essentiality utilising two pathogens in co-infection models identified ∼200 community-dependent essential genes with altered essentiality compared with monoculture [174]. Infection is inherently heterogeneous; therefore, the properties of antibacterial drug targets must be characterised under multiple conditions which recapitulate fundamental infection processes.

Chemical biology: bridging the gap between genetics and treatment

A sustainable supply of antibacterial drugs requires the integration of chemistry with biology to uncover new sources of bioactive compounds, to manipulate scaffolds to evade resistance mechanisms like efflux and enzymatic inactivation, and to rationally compose regimens which anticipate and suppress resistance. Historically, and very successfully, natural products were extracted from environmental isolates and simply tested for their ability to kill bacteria, but in the regime of diminishing returns we have encountered a bottleneck in natural product discovery. Now, emerging platforms provide purchasable, ultra-large, synthetically accessible chemical spaces of tens of billions which are impossible to test exhaustively, even *in silico* [175]. Facing the ambitious endeavour to find new molecules in nature or in the laboratory which are active against pathogenic bacteria, the new capabilities furnished by modern chemical biology and cheminformatics can be brought to bear to develop therapeutics science and prospect for chemical modulators of new targets which circumvent prevalent resistance mechanisms.

New chemical space: engaging new targets

Recent approaches to natural product prospecting demonstrate a widening of the discovery bottleneck. Making culturable the once-unculturable soil microbes has yielded promising new antibacterial candidates — including teixobactin [176], clovibactin [177], and darobactin [178] — within 9 years, demonstrating that sampling a fresh region of chemical space can readily yield new antibacterial compounds (Figure 4A).

To explore natural product-inspired chemistry, and to circumvent low cultivability of producers or low expression of native biosynthetic pathways which hamper natural product discovery [179], synthetic biologists first engineered existing natural product biosynthesis pathways in 1985, isolating new compounds mederrhodin and dihydrogranatirhodin from strains carrying combinations of the actinorhodin, granaticin, and medermycin biosynthetic pathways [180]. Over the last 40 years, revolutions in molecular biology and computational techniques allowed identification of new biosynthetic enzymes and engineering of known enzymes, manipulation of assembly-line order and composition, diversification of substrates, and optimisation of heterologous host strains [181–183]. However, re-discovery remains a problem despite synthetic biology and combinatorial

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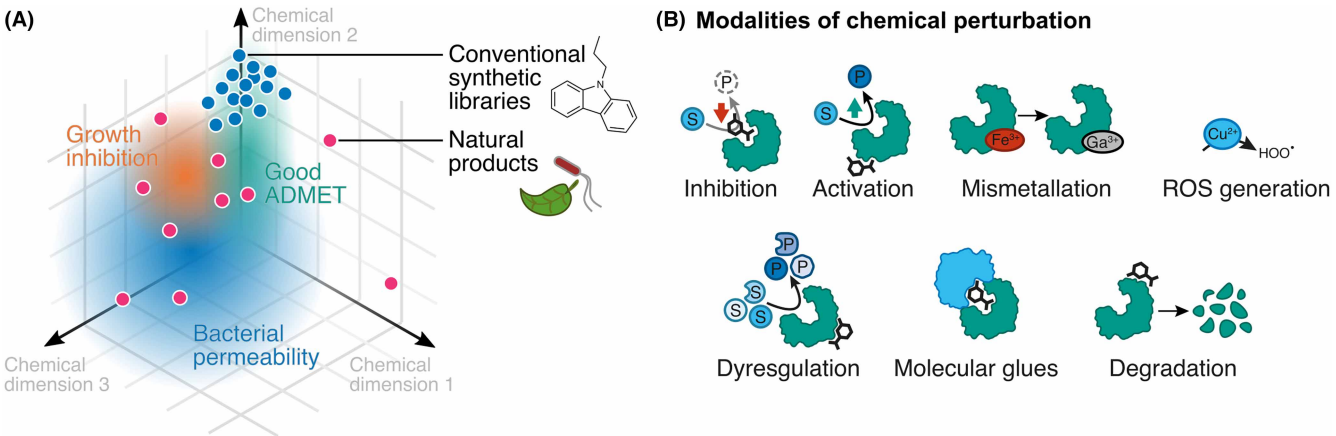

Figure 4. Exploring chemical space for bioactivity and new mechanisms of action.

(**A**) Schematic of a region of chemical space including bioactive compounds. The axes indicate dimensions of chemical space, the coloured clouds indicate areas with properties important for antibacterial drugs, and the coloured dots indicate compounds. Only a subset of compounds will permeate bacterial cells, while a minority of these will inhibit bacterial growth while demonstrating good absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties. Conventional combinatorial synthetic libraries (blue dots) cover a narrow chemical space and have been curated for human cell efficacy; therefore, few have found application as antibacterials. Many natural products (magenta dots) cover a wider chemical space and were curated by microbial antagonism for antibacterial efficacy. Synthetic libraries covering wider chemical space could be also artificially curated to prioritise bacterial cell entry, enriching for antibacterial activity. (**B**) An individual target could be modulated by a small molecule agent in a variety of ways, depending on the agent's specific structure.

chemistry approaches to modify natural products; therefore, the potential for pre-existing environmental resistance remains [15,178,183–189].

But untapped regions of chemical space need not necessarily be natural products, as testified by antituberculosis approvals and recent studies screening synthetic cyclic peptides, which readily yielded new clinical candidates like zosurabalpin (RG6006, in Phase I trials) [190]. Peptides and their derivatives represent a further constrained chemical space which can be explored densely if not exhaustively, as exemplified by recent discovery of complestatin, representing a new glycopeptide class with a novel mechanism of action. Another strategy seeks increased antibacterial potency through incorporation of metal-binding motifs into existing antimicrobial peptides such as anoplin [190–192], where the membrane-disrupting action was potentiated by the generation of reactive oxygen species by complexed copper, resulting in membrane lipid damage [193]. This mechanism is reminiscent of the natural product antibiotic and anticancer agent bleomycin, which binds iron and nucleic acids to cleave DNA and RNA through reactive oxygen species generation, but is too toxic to be used as an antibacterial drug. However, with a better understanding of the medicinal chemistry of organometallic antibiotics, the specificity of drugs like bleomycin might be tuned to selectively kill bacteria [194], perhaps by engineering selective permeation of bacterial cells.

Bacterial permeability and ADMET: reaching new targets

The subcellular location — cytoplasm, periplasm, or within the outer membrane — of a target also plays a role in its attractiveness. While Gram-positive bacteria possess a single membrane and a thick peptidoglycan layer, Gram-negative bacteria possess two membranes, separated by the periplasm and a thin peptidoglycan layer that must be traversed by a compound to reach a cytoplasmic target [195]. In general, targets on the outer membrane or in the periplasm, such as glycopeptides and penicillin-binding proteins, are more easily accessible [191,196–198]. Illustrating this phenomenon, accessible targets, like MmpL3 and DprE1, are over-represented from whole-cell *M. tuberculosis* screening studies due to their localisation on the membrane and the tendency towards hydrophobicity of screening libraries [199]. Some target redundancy accounts for attrition in the preclinical pipeline and can effect multiple mechanisms of action through the same target [152], but target diversity is also desirable although out of reach without sampling new regions of chemical space. Libraries designed with permeability in mind would expand the definition of chemically vulnerable targets.

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To this end, studies measuring compound accumulation in bacterial cells attempted to define the chemical properties required for bacterial cell uptake. While these properties vary according to envelope structure and mechanism of entry, understanding of some of them has enabled modification of existing Gram-positive antibiotics into compounds potent against Gram-negative bacteria [200–209]. This work also highlighted that even within Gram-negative bacteria and among strains of the same species there are large differences in the cell envelope, making cell entry rules species-specific [210,211]. While the resulting rules were applied to dialling-in Gram-negative activity of fabimycin [212], a proof-of-principle screen of a compound library curated from proprietary collections using these rules did not appear to yield any hits which could be progressed [213], indicating that there is probably still more to understand about bacterial cell entry by small molecules. An alternative approach to permeability is the Trojan horse strategy, originating with natural product antibiotic-siderophore conjugates like albomycin that chelate iron and are taken up by bacterial iron transporters to generate high intracellular antibiotic concentrations [214,215]. This approach has seen clinical approval with cefiderocol, a cephalosporin-catechol conjugate, and has also been used to sensitise the Gram-negative *Acinetobacter baumannii* to the Gram-positive antibiotic daptomycin by conjugation to a siderophore mimic [216,217].

However, permeation into the bacterial cell is only the first step towards clinical efficacy of a small molecule. For example, during tuberculosis infection, granulomas can shelter *M. tuberculosis* from isoniazid [218], while the partitioning of bedaquiline into lipid droplets in macrophages boosts its antituberculosis activity [219]. Antibacterial drugs are also subject to similar absorption, distribution, metabolism, excretion, and toxicity (ADMET) requirements as other therapeutics, with some additional considerations on toxicity and route of administration. Antibacterial drugs are usually taken at high doses over the course of a few weeks, except for the treatment of diseases caused by mycobacteria, such as tuberculosis (6–18 months) and leprosy (6–12 months), and prophylaxis for immunocompromised patients, which uses lower, long-term dosing. In the present paradigm of 'use it and lose it', new antibacterial drugs will typically be used as a last resort, so that some toxicity is tolerated by regulators. For example, the use of colistin, which is nephrotoxic, was abandoned in the late 20th century, but its use was revived in the early 21st century as a drug of last resort [220]. However, in low-resource settings and for diseases with long-term treatments, supportive care to complement toxic therapies may not be accessible, making toxicity is a more pressing consideration. The difference in ADMET requirements between antibacterial drugs and drugs for diseases not caused by bacteria can be illustrated where they intersect, for example with doxorubicin, an intravenous topoisomerase inhibitor which causes immunogenic cell death in cancer at in the low nanomolar range, and which also kills mycobacteria in the low micromolar range [221]. It is barely tolerated as an antineoplastic agent, and probably would never be used in its present form as an antituberculosis drug.

Similarly, desirable routes of administration also vary by disease, the plurality of alternative therapeutics, and availability of clinical resources. For example, intravenous dosing often avoids first-pass metabolism, but it requires expert attention which is not always available in primary care. In contrast, oral dosing requires greater investment in medicinal chemistry optimisation, and, for a given chemical scaffold engaging a promising target, antibacterial activity and oral bioavailability may be mutually exclusive. Therefore, when assembling chemical libraries for phenotypic screening, it is essential to curate scaffolds which are predicted to be or a short derivatisation from being orally bioavailable.

Alongside considering host metabolism, bacterial metabolism can also present opportunities and challenges to medicinal chemistry. Enzymes which modify antibacterial candidates can inactivate them, or in the case of prodrugs, activate them. Such activating modifications can allow an inactive-but-permeable chemical species to reach the bacterial cytoplasm and acquire an active-but-impermeable form which accumulates inside the cell, as readily visualised for the dye calcein acetoxymethyl ester [222], and as is the case for the antituberculosis prodrug isoniazid [223]. Some competitive inhibitors of two-substrate enzymes, such as dihydropteroate synthase inhibitor sulfamethoxazole, also act through a secondary mechanism of substrate wasting where the drug forms an adduct with an essential metabolite, thus depleting it. This mechanism precludes resistance through target amplification, since overexpression only exacerbates this substrate wasting [98]. As demonstrated with *para*-aminosalicylic acid, the drug-metabolite adduct can also inhibit enzymes downstream of the parent drug's target [99], increasing the barrier to resistance.

Beyond inhibition: effecting new phenotypes through chemistry

The ability to bind small molecules to a target is a basic yet important requirement for target selection [224]. Many antibiotics, like penicillin, achieve target inhibition by binding to an enzyme's active site, but others bind

elsewhere on their target protein, like rifampicin which binds to the DNA/RNA channel of RNA polymerase to block the path of elongating RNA [225,226]. Compounds with non-traditional modalities expand our ability to modulate targets beyond simple inhibition, such as the activation and dysregulation of ClpP by ADEP and ACP compounds, or over-activation of DegP by tripodal peptidyl compounds (Figure 4B) [227–229]. Pyrazinoic acid, the active form of the first-line tuberculosis antibiotic pyrazinamide, has been shown to trigger degradation of PanD rather than traditional functional inhibition [24]. The recent development of BacPROTACs offers a generalisation of this strategy, since they reprogramme the ClpCP system in Gram-positive bacteria to achieve specific degradation of proteins [97].

Metal-based compounds offer a range of alternative mechanisms of action, for example, mismetallation of iron-dependent enzymes by gallium complexes results in growth inhibition and biofilm disruption (Figure 4B) [192,193,230,231]. Molecular glues cause non-natural interactions between biomolecules, but none have been discovered for bacterial pathogens, despite the use of thalidomide — a cereblon ligand often used to design proteolysis targeting chimaeras in human cells — since the 1960s as a host-directed therapy to treat complications of leprosy, caused by *Mycobacterium leprae* [232].

Genetically encoded targets are not the only possible targets within a cell, demonstrated by glycopeptides such as vancomycin, ramoplanin, and complestatin which bind cell wall components to prevent cross-linking, transglycosylation, and cell wall remodelling [191,233,234]. Non-encoded targets such as lipids and glycans represent an underutilised class; therefore, new techniques are needed to evaluate them as antibacterial drug targets. Chemical probes to study and modulate both genetically encoded and non-encoded targets will prove invaluable, such as use of trehalose analogues to study pathway-dependent labelling of mycobacterial glycolipids, or study of post-translational modification through bump-and-hole engineering, recently used to identify substrate specificities of particular isoenzymes *in vivo* through engineered glycosyltransferases and tagged glycans [235–237].

Unlocking the third wave of antibacterial drug discovery

To sustain the repertoire of antibacterial drugs with clinical utility, discovery of non-toxic, potent small molecules with new mechanisms of action must outpace emergence of new resistance. This challenge can be met with the integration between chemistry and genetics, as highlighted by recent efforts in exploring the antibacterial chemical space, deriving resistance-proof natural products, and anticipating new resistance mechanisms.

Prioritising unexplored chemical space for likely bioactivity

The ADMET criteria for drugs to treat bacterial infections are distinct from those to treat diseases like cancer and diabetes. Since the vast chemical libraries of large pharmaceutical companies are biased towards the ADMET and cell permeability properties for activity in human cells, it is possible that the apparent failure of target-based antibacterial drug discovery — where compounds active *in vitro* failed to penetrate the bacterial cell wall — was caused more by unsuitable chemical matter than by flawed methodology. Decades of research on human diseases in general has defined heuristics to guide curation of suitable chemical libraries [238–240], but there are no such comprehensive guides for antibacterial drugs, and worse, some properties which are broadly considered unfavourable are the definition of success for some of the best antibacterial drugs. For example, nitrofurantoin is both reactive and possesses an aromatic nitro group, properties which would trigger structural alerts and exclude it from most typical chemical libraries but which also drive its efficacy.

The heuristics for curating antibacterial drug discovery libraries will have some overlap with the current rules. Therapeutic index, the gap between therapeutic and toxic doses, must be more conservative, whereas oral bioavailability could be dispensable in the most dire clinical cases. To unlock the intracellular majority of essential targets, chemical libraries optimised for phenotypic screening must be enriched for compounds which penetrate the bacterial cell wall, while those for target-based discovery should contain compounds within a few robust synthetic steps from bacterial permeability. To curate such libraries, reliable models of ADMET properties and bacterial permeability are necessary.

While favourable ADMET properties and bacterial permeability are necessary for antibacterial drugs, they are not sufficient. Unlike other diseases caused by one or a handful of gene products, any essential gene product or other non-encoded biomolecule in bacterial pathogens is a vulnerability, collectively presenting a large surface area for targeting. Whereas antibacterial drug discovery has historically focussed on natural products and therefore new discovery has been stymied by the coupon collector problem, recent works which sampled underexplored chemical space have readily discovered bioactive compounds.

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Defining a new but constrained chemical space to a region expected to be enriched in bioactivity enables systematic, thorough exploration (Figure 5A). For example, zosurabalpin was discovered through a medium-sized screen of 44 985 synthetic macrocyclic peptides [190], a relatively unexplored chemical class. Similarly, new sources of natural products can also yield very high hit rates. For example, bioinformatic identification of biosynthetic gene clusters from the human microbiome followed by their heterologous expression in *E. coli* yielded a handful of new antimicrobial peptides from 70 candidates [241].

Sampling new chemical space has also been directed by strategies employing deep learning. In a recent thread of work, a message-passing neural network was trained on high quality data representing the inhibitory activity of a small set of 2335 unique molecules comprising FDA-approved drugs and natural products, of which 120 had inhibitory activity against *E. coli* [242]. Given the small number of positive training examples, the large number of potential mechanisms of action represented by the hundreds of essential genes in *E. coli*, and the combinatorial vastness of potential chemical features beyond the scaffolds of known antibiotics, the model likely learned to recognise some features of *E. coli* permeability. The trained model was used to curate

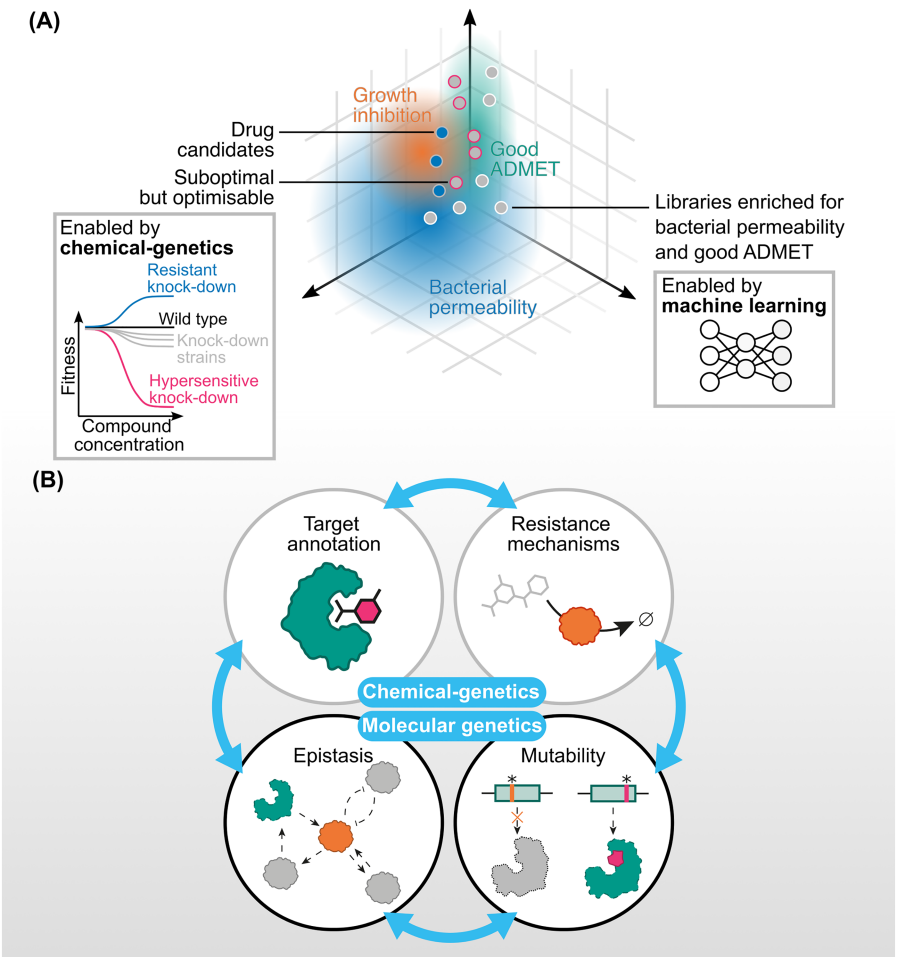

Figure 5. Strategies to unlock the third wave of antibacterial therapies.

(**A**) Schematic of screening chemical libraries enriched for bacterial permeability and good ADMET. With appropriate predictors of bacterial permeability and ADMET, potentially furnished by machine learning models, new chemical libraries could be designed with these properties in mind. Using chemical–genetic assays like PROSPECT allow sensitive detection of compounds that are not active against wild-type bacteria but do affect knockdown strains, enabling a sparser search of chemical space and therefore accelerating the identification of new bioactive chemical scaffolds. These scaffolds can then be optimised for wild-type activity. (**B**) Chemical–genetics and molecular genetics provide complementary tools for anticipating resistance and for designing combination regimens which suppress or exploit it.

23 molecules from the ZINC15 database of >100 million purchasable or readily synthesisable compounds, which might not otherwise have been screened against bacterial pathogens. This approach yielded two compounds with appreciable activity against *E. coli*, demonstrating the potential of sampling new chemical space for antibacterial candidates. Later, the same model architecture was trained on a dataset containing 7684 compounds including 480 actives against the priority pathogen *A. baumanii*, ultimately yielding abaucin [243]. More recent work enriched chemical spaces with favourable ADMET by using models to score both human cytotoxicity and antibacterial potency, trained on 512 positives out of 39 312 compounds screened against *S. aureus*, enabling rapid prioritisation of compounds for phenotypic testing and yielding promising new antibacterial candidates [244].

Even with these computational methods, scoring the ever-expanding subset of readily synthesisable compounds, currently ∼50 billion, is resource-intensive, and out of reach of computational docking screens [245], which are currently limited to hundreds of millions of compounds, although methods like V-SYNTHES which operate in lower-dimensionality reagent space [175] provide a means to sift through enormous virtual libraries. Such tools that enable a sparse search of chemical space will be invaluable to prospect for bioactivity, which can then be mined using chemically focused libraries. To this end, active learning is an emerging framework to iteratively screen subsets of vast chemical libraries [246,247]. By training deep learning models equipped with a measure of uncertainty or variance in their predictions, smaller compound libraries can be enriched with compounds predicted to be high activity with low uncertainty. Alternatively, to improve the model's generalisability to compounds dissimilar to the training set, the next library can contain compounds annotated with maximal uncertainty, so that the updated model can learn from their data. In this way, advanced statistical models can steer a data-driven search for new antibacterial activity.

To enable a sparse search of chemical space in the wet laboratory, bacterial mutants with essential genes knocked down can be used as sentinels of suboptimal but optimisable chemical space. Because essential gene knockdown mutants are hypersensitive to inhibitors of pathways related to their knocked down gene product, their growth is inhibited either at lower concentrations or lower potencies. For example, using the PROSPECT assay, it was possible to identify the inhibitor BRD-8000, which had no measurable activity against wild-type *M. tuberculosis* but inhibited the growth of the *efpA* knockdown [129]. The dimethylcyclopropanyl scaffold of BRD-8000 resembled neurotoxic chrysanthemic acid derivatives used as insecticides, but the specific activity of the compound against a mutant encouraged structure–activity relationship studies which revealed that the (S, S)-enantiomer of BRD-8000 was active, in contrast with the insecticidal (R,R)-enantiomer of chrysanthemic acid. Further medicinal chemistry led to an inhibitor with micromolar potency against wild-type *M. tuberculosis*, demonstrating that unusual or even unattractive chemical space can contain chemical modulators of new targets with phenotypic activity. As with all new antibacterial agents, it will be essential to understand the resistance liabilities of these new mechanisms of action.

Anticipating and exploiting resistance

Resistance to antibacterial drugs is empirically inevitable with present agents, even when resistance appeared unlikely. For example, since the cell wall tripeptide target of tricyclic glycopeptide vancomycin is not encoded, resistance was thought to be vanishingly unlikely. However, although no vancomycin resistance was detected decades after its discovery in 1957, its eventual clinical deployment in the 1970s ushered in high-level resistance in *Enterococcus* 16 years later [248], a similar time-to-resistance to other antibacterial drugs (Figure 1B). The main resistance mechanisms are carried on a transposable element which confers vancomycin sensing and subsequent biosynthesis of cell wall tripeptides with lower vancomycin affinity [249].

Thus, there should be no complacency with new antibacterial agents about the emergence of resistant isolates, even when it does not easily evolve in the laboratory where, conventionally, high-density bacterial cultures are plated on *supra*-inhibitory compound concentrations. While this approach is often successful, it is prone to false negatives, partly because it focusses on spontaneous mutation at a rate higher than $\sim 10^{-9}$ within a monoculture (excluding horizontal gene transfer from the environment or host microbiome), partly because drug concentrations at infection sites are not necessarily constant and *supra*-inhibitory, enabling a stepwise evolutionary path through low-level resistance. For example, *E. coli* have demonstrated the ability to acquire mutations that confer tolerance — slowed death in the presence of normally lethal ampicillin concentrations — which in turn bought time for acquisition of high-level resistance alleles [250]. A similar phenomenon was described in *Mycobacterium smegmatis*, where ribosomal mutations with a fitness cost enabled low-level broad-spectrum resistance followed by high-level resistance and compensatory mutations to restore wild-type growth rates [251].

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Additionally, mutation rates during infection may be higher than in broth, as demonstrated by higher rate of rifampicin resistance in cultures derived from patients than those from an axenic culture. Random mutations may also be distributed across the genome differently during infection, since an important mechanism of mutagenesis is replication–transcription conflict [151], where actively transcribed genes orientated against the direction of replication cause stalled replisome and DNA damage, leading to mutation. Since the transcriptional activity of bacteria during infection is distinct from that in axenic culture, it is likely that there are spontaneous mutants resulting in resistance not observed *in vitro*.

As a result, resistance to antibacterial drugs is usually a surprise, Fleming's warnings in 1945 notwithstanding. To be better prepared, recent work has applied deep mutational scanning to exhaustively quantify the effects of target mutations on drug resistance and fitness in DHFR in *E. coli* [252] and PncA, the activating enzyme of pyrazinamide, in *M. tuberculosis* [253]. Expanded efforts across genomes of pathogens combined with determination of infection fitness, collateral sensitivity to existing drugs, and interactions with genetic perturbations modelling undiscovered agents would enable anticipating resistance and exploiting it, potentially with designed combination therapies (Figure 5B).

From agents to regimens: rationally designing optimal combination therapies

The most successful antibacterial drugs, the β-lactams, are multi-target inhibitors to which spontaneous resistance is rare. Instead, resistance to these agents is usually acquired only through mobile genetic elements like plasmids or transposons [146]. Whilst engineering synthetic antibiotics to target multiple sites is challenging, examples under investigation include repurposed antirheumatic agent auranofin and antimycobacterial agent BB2-50F, which inhibits ATP synthase and succinate dehydrogenase [254–258].

Nevertheless, resistance to these inhibitors does occur; therefore, some efforts focus on preserving and renewing the utility of the current antibiotic repertoire, rather than identifying new molecules. Such adjuvants aim to tackle existing resistance or extend the range of activity of a drug [259–261]. Although only β-lactamase inhibitors are approved adjuvants, there are several under investigation with diverse mechanisms: class I adjuvants directly inhibit (e.g. β-lactamase and efflux inhibitors) or bypass resistance (e.g. teichoic acid synthesis inhibitors and membrane-disruptors), whereas class II adjuvants modulate host processes to increase bacterial killing [262–267]. Other adjuvants increase the working spectrum of antibiotics, by sensitising Gram-negative organisms to Gram-positive antibiotics [268,269].

Another method of prospectively supressing resistance is to combine antibacterial drugs. For example, since the 1950s, the tuberculosis standard of care comprises drugs mostly with single targets and *in vitro* resistance frequencies between 10^{-9} and 10^{-6} [12,270], requiring a combination of four drugs to suppress evolution of resistance. The combination strategy also presents the opportunity to exploit synergistic interactions [12,271–273], which have an additional benefit that, whereas some synergies are broadly conserved, others are species-selective, thus generating narrow-spectrum therapies that minimise harm to the host or their microbiome [267,274,275].

Other combinatorial strategies use synthetic lethal interactions, whereby inhibition of two non-essential gene products, sometimes by a single agent, results in bacterial killing. For example, both MurA1 and MurA2, which are genetically redundant and individually non-essential in Gram-positive *S. pneumoniae* and *S. aureus*, are inhibited by fosfomycin and other synthetic agents, ultimately killing the bacteria [276–281]. However, synthetically lethal therapies are vulnerable to resistance to either drug rendering the combination ineffective, as demonstrated by development of resistance to cancer therapies utilising synthetic lethal interactions between BRCA2 mutants and PARP inhibitors or cisplatin through secondary BRCA2 mutation [282,283]. A strategic approach that exploits resistance development couples drugs with collateral sensitivity, whereby evolution of resistance to one drug renders the bacterium hypersensitive to the second [152,284,285]. To design such strategies, it is essential to understand the structure of underlying genetic interaction networks, and the consequent effects of chemical perturbation.

In an example of this combinatorial paradigm, trimethoprim is usually paired with sulfamethoxazole (a combination known as co-trimoxazole), which inhibits dihydropteroate synthase, an enzyme upstream of DHFR in the folic acid biosynthesis pathway. While both drugs are effective alone, they are synergistic in combination. Knowledge of these compounds' targets and their network biology (Figure 2B) retrospectively explained the efficacy of co-trimoxazole [286]. Continued development of our understanding of antibacterial targets and bacterial physiology will empower future proactive design of optimal regimens.

Understanding of the target of trimethoprim and DHFR's network context has also revealed resistance liabilities for drugs targeting this essential enzyme. For example, loss-of-function mutations in thymidylate synthase

render DHFR dispensable and drugs inhibiting it ineffective [133], or up-regulated RibD, a riboflavin biosynthesis protein, can moonlight as DHFR to enable bacterial survival in the presence of *para*-aminosalicylic acid (Figure 2B) [287]. As new small molecules with new mechanisms of action are discovered, understanding their systems chemical biology will be critical to anticipate and exploit resistance.

Conclusion

Fundamental pathogen biology in relevant infection models leads to the identification and evaluation of putative targets of new antibacterial candidates. There are a range of techniques with unique advantages and disadvantages, often depending on the target under investigation. However, defining targets solely from a biological perspective is not sufficient: it is important to consider whether these targets can be modulated through chemical approaches, what type of modulation can be achieved, and if compounds capable of achieving these modulations can access the subcellular location within which the target resides. Therefore, understanding which areas of chemical space possess these properties will help either reduce the size of chemical screens for target activity, or increase the number of hits which provide starting material to develop lead candidates.

The vastness of this task can be aided by leveraging deep learning to prioritise candidates from ultra-large synthesis-on-demand chemical spaces [288]. Emerging genome-scale genetic techniques such as deep mutational scanning and metagenome complementation will assist medicinal chemists by prospectively identifying resistance liabilities both of target and chemical class. Alongside molecular genetics in the laboratory, it will be critical to consider the population genomics of pathogens and hosts to define the landscape of natural alleles which will influence the assessment of likely activity and resistance of any given antibacterial regimen. In this way, as a companion to new therapeutics, rapid diagnostics which determine the best course of therapy for a given infection will have an important role, enabled by molecular and population genetics. As host-directed strategies develop for bacterial infections, human molecular genetics and chemical biology will aid their clinical implementation.

Ultimately, integrating molecular genetics with chemical biology will allow exploration of target or compound combinations that possess collateral sensitivity, synergy, or combat likely resistance mechanisms to realise the full potential of new and old antibacterial agents. More than a century after discovering the cause of tuberculosis using methylene blue, a new wave of therapies can be inspired by the integrative chemical biology approach of 'thinking chemically and acting biologically' [289].

Data Availability

Code and data used to generate Figure 1 are available at GitHub (https://github.com/scbirlab/2024-Parkhill-BiochemJ).

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Eachan O. Johnson: Conceptualisation, Supervision, Funding acquisition, Writing — review and editing.
Susannah L. Parkhill: Conceptualisation, Writing — original draft.

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References

1 Murray, C.J., Ikuta, K.S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A. et al. (2022) Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* **399**, 629–655 https://doi.org/10.1016/S0140-6736(21)02724-0
2 O'Neill, J. (2016) Tackling drug-resistant infections globally: Final report and recommendations. https://amr-review.org/

Biochemical Journal (2024) **481** 839–864
https://doi.org/10.1042/BCJ20220062

3 Stennett, H.L., Back, C.R. and Race, P.R. (2022) Derivation of a precise and consistent timeline for antibiotic development. *Antibiotics* **11**, 1237 https://doi.org/10.3390/antibiotics11091237
4 Payne, D.J., Gwynn, M.N., Holmes, D.J. and Pompliano, D.L. (2006) Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nat. Rev. Drug Discov.* **6**, 29–40 https://doi.org/10.1038/nrd2201
5 Sadri, A. (2023) Is target-based drug discovery efficient? Discovery and 'off-target'; mechanisms of all drugs. *J. Med. Chem.* **66**, 12651–12677 https://doi.org/10.1021/acs.jmedchem.2c01737
6 Aminov, R.I. (2010) A brief history of the antibiotic era: lessons learned and challenges for the future. *Front. Microbiol.* **1**, 134. https://doi.org/10.3389/fmicb.2010.00134
7 Wright, P.M., Seiple, I.B. and Myers, A.G. (2014) The evolving role of chemical synthesis in antibacterial drug discovery. *Angew. Chem. Int. Ed.* **53**, 8840–8869 https://doi.org/10.1002/anie.201310843
8 Chain, E., Florey, H.W., Adelaide, M.B., Gardner, A.D., Heatley, N.G., Jennings, M.A. et al. (1940) Penicillin as a chemotherapeutic agent. *Lancet* **236**, 226–228 https://doi.org/10.1016/S0140-6736(01)08728-1
9 Walsh, C. (2003) Where will new antibiotics come from? *Nat. Rev. Microbiol.* **1**, 65–70 https://doi.org/10.1038/nrmicro727
10 Fischbach, M.A. and Walsh, C.T. (2009) Antibiotics for emerging pathogens. *Science* **325**, 1089–1093 https://doi.org/10.1126/science.1176667
11 Patridge, E., Gareiss, P., Kinch, M.S. and Hoyer, D. (2016) An analysis of FDA-approved drugs: natural products and their derivatives. *Drug Discov. Today* **21**, 204–207 https://doi.org/10.1016/j.drudis.2015.01.009
12 Medical Research Council. (1950) Treatment of pulmonary tuberculosis with streptomycin and para-amino-salicylic acid. *Br. Med. J.* **2**, 1073–1085 https://doi.org/10.1136/bmj.2.4688.1073
13 Cohen, S.S. (1977) A strategy for the chemotherapy of infectious disease. *Science* **197**, 431–432 https://doi.org/10.1126/science.195340
14 Tommasi, R., Brown, D.G., Walkup, G.K., Manchester, J.I. and Miller, A.A. (2015) ESKAPEing the labyrinth of antibacterial discovery. *Nat. Rev. Drug Discov.* **14**, 529–542 https://doi.org/10.1038/nrd4572
15 Stapley, E.O. (1958) Cross-resistance studies and antibiotic identification. *Appl. Microbiol.* **6**, 392–398 https://doi.org/10.1128/am.6.6.392-398.1958
16 Baltz, R.H. (2006) Marcel Faber Roundtable: is our antibiotic pipeline unproductive because of starvation, constipation or lack of inspiration? *J. Ind. Microbiol. Biotechnol.* **33**, 507–513 https://doi.org/10.1007/s10295-005-0077-9
17 Butler, M.S., Henderson, I.R., Capon, R.J. and Blaskovich, M.A.T. (2023) Antibiotics in the clinical pipeline as of December 2022. *J. Antibiot. (Tokyo)* **76**, 431–473 https://doi.org/10.1038/s41429-023-00629-8
18 U.S. Food and Drug Administration. (2023) FDA Approves New Treatment for Pneumonia Caused by Certain Difficult-to-Treat Bacteria https://www.fda.gov/news-events/press-announcements/fda-approves-new-treatment-pneumonia-caused-certain-difficult-treat-bacteria
19 US Food and Drug Administration. (2019) FDA approves new drug for treatment-resistant forms of tuberculosis that affects the lungs https://www.fda.gov/news-events/press-announcements/fda-approves-new-drug-treatment-resistant-forms-tuberculosis-affects-lungs
20 Otsuka Pharmaceutical Co. Ltd. (2014) Otsuka Wins European Marketing Authorization for Deltyba: (delamanid) https://www.otsuka.co.jp/en/company/newsreleases/2014/20140430_1.html
21 Butler, M.S. and Cooper, M.A. (2011) Antibiotics in the clinical pipeline in 2011. *J. Antibiot. (Tokyo)* **64**, 413–425 https://doi.org/10.1038/ja.2011.44
22 Lewis, K. (2020) The science of antibiotic discovery. *Cell* **181**, 29–45 https://doi.org/10.1016/j.cell.2020.02.056
23 Shi, W., Zhang, X., Jiang, X., Yuan, H., Lee, J.S., Barry, C.E. et al. (2011) Pyrazinamide inhibits trans-translation in *Mycobacterium tuberculosis*. *Science* **333**, 1630–1632 https://doi.org/10.1126/science.1208813
24 Gopal, P., Sarathy, J.P., Yee, M., Ragunathan, P., Shin, J., Bhushan, S. et al. (2020) Pyrazinamide triggers degradation of its target aspartate decarboxylase. *Nat. Commun.* **11**, 1661 https://doi.org/10.1038/s41467-020-15516-1
25 Kacser, H. and Burns, J.A. (1981) The molecular basis of dominance. *Genetics* **97**, 639–666 https://doi.org/10.1093/genetics/97.3-4.639
26 Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M. et al. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2**, 2006.0008 https://doi.org/10.1038/msb4100050
27 Koo, B.M., Kritikos, G., Farelli, J.D., Todor, H., Tong, K., Kimsey, H. et al. (2017) Construction and analysis of two genome-scale deletion libraries for *Bacillus subtilis*. *Cell Syst.* **4**, 291–305.e7 https://doi.org/10.1016/j.cels.2016.12.013
28 Porwollik, S., Santiviago, C.A., Cheng, P., Long, F., Desai, P., Fredlund, J. et al. (2014) Defined single-gene and multi-gene deletion mutant collections in *Salmonella enterica* sv Typhimurium. *PLoS One* **9**, e99820 https://doi.org/10.1371/journal.pone.0099820
29 De Berardinis, V., Vallenet, D., Castelli, V., Besnard, M., Pinet, A., Cruaud, C. et al. (2008) A complete collection of single-gene deletion mutants of *Acinetobacter baylyi* ADP1. *Mol Syst Biol* **4**, 174 https://doi.org/10.1038/msb.2008.10
30 Wei, D., Wang, M., Shi, J. and Hao, J. (2012) Red recombinase assisted gene replacement in *Klebsiella pneumoniae*. *J. Ind. Microbiol. Biotechnol.* **39**, 1219–1226 https://doi.org/10.1007/s10295-012-1117-x
31 Murphy, K.C., Nelson, S.J., Nambi, S., Papavinasasundaram, K., Baer, C.E. and Sassetti, C.M. (2018) Orbit: a new paradigm for genetic engineering of mycobacterial chromosomes. *mBio* **9**, e01467-18 https://doi.org/10.1128/mBio.01467-18
32 van Opijnen, T., Bodi, K.L. and Camilli, A. (2009) Tn-seq: high-throughput parallel sequencing for fitness and genetic interaction studies in microorganisms. *Nat. Methods* **6**, 767–772 https://doi.org/10.1038/nmeth.1377
33 Langridge, G.C., Phan, M.D., Turner, D.J., Perkins, T.T., Parts, L., Haase, J. et al. (2009) Simultaneous assay of every Salmonella Typhi gene using one million transposon mutants. *Genome Res.* **19**, 2308–2316 https://doi.org/10.1101/gr.097097.109
34 Gawronski, J.D., Wong, S.M.S., Giannoukos, G., Ward, D.V. and Akerley, B.J. (2009) Tracking insertion mutants within libraries by deep sequencing and a genome-wide screen for Haemophilus genes required in the lung. *Proc. Natl Acad. Sci. U.S.A.* **106**, 16422–16427 https://doi.org/10.1073/pnas.0906627106
35 Goodman, A.L., McNulty, N.P., Zhao, Y., Leip, D., Mitra, R.D., Lozupone, C.A. et al. (2009) Identifying genetic determinants needed to establish a human gut symbiont in its habitat. *Cell Host Microbe* **6**, 279–289 https://doi.org/10.1016/j.chom.2009.08.003
36 Wetmore, K.M., Price, M.N., Waters, R.J., Lamson, J.S., He, J., Hoover, C.A. et al. (2015) Rapid quantification of mutant fitness in diverse bacteria by sequencing randomly bar-coded transposons. *mBio* **6**, e00306 https://doi.org/10.1128/mBio.00306-15
37 Jacobs, M.A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S. et al. (2003) Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc. Natl Acad. Sci. U.S.A.* **100**, 14339–14344 https://doi.org/10.1073/pnas.2036282100

38 Liberati, N.T., Urbach, J.M., Miyata, S., Lee, D.G., Drenkard, E., Wu, G. et al. (2006) An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc. Natl Acad. Sci. U.S.A.* **103**, 2833–2838 https://doi.org/10.1073/pnas.0511100103
39 Ramage, B., Erolin, R., Held, K., Gasper, J., Weiss, E., Brittnacher, M. et al. (2017) Comprehensive arrayed transposon mutant library of *Klebsiella pneumoniae* outbreak strain KPNIH1. *J. Bacteriol.* **199**, e00352-17 https://doi.org/10.1128/JB.00352-17
40 Geoffroy, M.C., Floquet, S., Métais, A., Nassif, X. and Pelicic, V. (2003) Large-scale analysis of the meningococcus genome by gene disruption: resistance to complement-mediated lysis. *Genome Res.* **13**, 391–398 https://doi.org/10.1101/gr.664303
41 Cameron, D.E., Urbach, J.M. and Mekalanos, J.J. (2008) A defined transposon mutant library and its use in identifying motility genes in *Vibrio cholerae*. *Proc. Natl Acad. Sci. U.S.A.* **105**, 8736–8741 https://doi.org/10.1073/pnas.0803281105
42 Gallagher, L.A., Ramage, E., Jacobs, M.A., Kaul, R., Brittnacher, M. and Manoil, C. (2007) A comprehensive transposon mutant library of *Francisella novicida*, a bioweapon surrogate. *Proc. Natl Acad. Sci. U.S.A.* **104**, 1009–1014 https://doi.org/10.1073/pnas.0606713104
43 Kumar, A., Des Etages, S.A., Coelho, P.S.R., Roeder, G.S. and Snyder, M. (2000) High-throughput methods for the large-scale analysis of gene function by transposon tagging. *Methods Enzymol.* **328**, 550–574 https://doi.org/10.1016/s0076-6879(00)28418-8
44 Goodall, E.C.A., Robinson, A., Johnston, I.G., Jabbari, S., Turner, K.A., Cunningham, A.F. et al. (2018) The essential genome of *Escherichia coli* K-12. *mBio* **9**, e02096-17 https://doi.org/10.1128/mBio.02096-17
45 Poulsen, B.E., Yang, R., Clatworthy, A.E., White, T., Osmulski, S.J., Li, L. et al. (2019) Defining the core essential genome of *Pseudomonas aeruginosa*. *Proc. Natl Acad. Sci. U.S.A.* **116**, 10072–10080 https://doi.org/10.1073/pnas.1900570116
46 Chaudhuri, R.R., Allen, A.G., Owen, P.J., Shalom, G., Stone, K., Harrison, M. et al. (2009) Comprehensive identification of essential *Staphylococcus aureus* genes using Transposon-Mediated Differential Hybridisation (TMDH). *BMC Genomics* **10**, 291 https://doi.org/10.1186/1471-2164-10-291
47 DeJesus, M.A., Gerrick, E.R., Xu, W., Park, S.W., Long, J.E., Boutte, C.C. et al. (2017) Comprehensive essentiality analysis of the *Mycobacterium tuberculosis* genome via saturating transposon mutagenesis. *mBio* **8**, e02133-16 https://doi.org/10.1128/mBio.02133-16
48 Jordan, I.K., Rogozin, I.B., Wolf, Y.I. and Koonin, E.V. (2002) Essential genes are more evolutionarily conserved than are nonessential genes in bacteria. *Genome Res.* **12**, 962–968 https://doi.org/10.1101/gr.87702
49 Goh, S., Boberek, J.M., Nakashima, N., Stach, J. and Good, L. (2009) Concurrent growth rate and transcript analyses reveal essential gene stringency in *Escherichia coli*. *PLoS One* **4**, e6061 https://doi.org/10.1371/journal.pone.0006061
50 Bosch, B., DeJesus, M.A., Poulton, N.C., Zhang, W., Engelhart, C.A., Zaveri, A. et al. (2021) Genome-wide gene expression tuning reveals diverse vulnerabilities of *M. tuberculosis*. *Cell* **184**, 4579–4592.e24 https://doi.org/10.1016/j.cell.2021.06.033
51 Alper, H., Fischer, C., Nevoigt, E. and Stephanopoulos, G. (2005) Tuning genetic control through promoter engineering. *Proc. Natl Acad. Sci. U.S.A.* **102**, 12678–12683 https://doi.org/10.1073/pnas.0504604102
52 Meynial-Salles, I., Cervin, M.A. and Soucaille, P. (2005) New tool for metabolic pathway engineering in *Escherichia coli*: one-step method to modulate expression of chromosomal genes. *Appl. Environ. Microbiol.* **71**, 2140–2144 https://doi.org/10.1128/AEM.71.4.2140-2144.2005
53 Nishino, K. and Yamaguchi, A. (2001) Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. *J. Bacteriol.* **183**, 5803–5812 https://doi.org/10.1128/JB.183.20.5803-5812.2001
54 Solem, C. and Jensen, P.R. (2002) Modulation of gene expression made easy. *Appl. Environ. Microbiol.* **68**, 2397–2403 https://doi.org/10.1128/AEM.68.5.2397-2403.2002
55 Stieger, M., Wohlgensinger, B., Kamber, M., Lutz, R. and Keck, W. (1999) Integrational plasmids for the tetracycline-regulated expression of genes in *Streptococcus pneumoniae*. *Gene* **226**, 243–251 https://doi.org/10.1016/s0378-1119(98)00561-7
56 Fan, F., Lunsford, R.D., Sylvester, D., Fan, J., Celesnik, H., Iordanescu, S. et al. (2001) Regulated ectopic expression and allelic-replacement mutagenesis as a method for gene essentiality testing in *Staphylococcus aureus*. *Plasmid* **46**, 71–75 https://doi.org/10.1006/plas.2001.1526
57 Zhang, L., Fan, F., Palmer, L.M., Lonetto, M.A., Petit, C., Voelker, L.L. et al. (2000) Regulated gene expression in *Staphylococcus aureus* for identifying conditional lethal phenotypes and antibiotic mode of action. *Gene* **255**, 297–305 https://doi.org/10.1016/s0378-1119(00)00325-5
58 Bikard, D., Jiang, W., Samai, P., Hochschild, A., Zhang, F. and Marraffini, L.A. (2013) Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Res.* **41**, 7429–7437 https://doi.org/10.1093/nar/gkt520
59 Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P. et al. (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **152**, 1173–1183 https://doi.org/10.1016/j.cell.2013.02.022
60 Vigouroux, A., Oldewurtel, E., Cui, L., Bikard, D. and vanTeeffelen, S. (2018) Tuning dCas9's ability to block transcription enables robust, noiseless knockdown of bacterial genes. *Mol. Syst. Biol.* **14**, e7899 https://doi.org/10.15252/msb.20177899
61 Li, X.T., Jun, Y., Erickstad, M.J., Brown, S.D., Parks, A., Court, D.L. et al. (2016) tCRISPRi: tunable and reversible, one-step control of gene expression. *Sci. Rep.* **6**, 39076 https://doi.org/10.1038/srep39076
62 Cui, L., Vigouroux, A., Rousset, F., Varet, H., Khanna, V. and Bikard, D. (2018) A CRISPRi screen in *E. coli* reveals sequence-specific toxicity of dCas9. *Nat. Commun.* **9**, 1912 https://doi.org/10.1038/s41467-018-04209-5
63 Rousset, F., Cabezas-Caballero, J., Piastra-Facon, F., Fernández-Rodríguez, J., Clermont, O., Denamur, E. et al. (2021) The impact of genetic diversity on gene essentiality within the *Escherichia coli* species. *Nat. Microbiol.* **6**, 301–312 https://doi.org/10.1038/s41564-020-00839-y
64 Cho, S., Choe, D., Lee, E., Kim, S.C., Palsson, B. and Cho, B.K. (2018) High-level dCas9 expression induces abnormal cell morphology in *Escherichia coli*. *ACS Synth. Biol.* **7**, 1085–1094 https://doi.org/10.1021/acssynbio.7b00462
65 Calvo-Villamañán, A., Ng, J.W., Planel, R., Ménager, H., Chen, A., Cui, L. et al. (2020) On-target activity predictions enable improved CRISPR–dCas9 screens in bacteria. *Nucleic Acids Res.* **48**, e64 https://doi.org/10.1093/nar/gkaa294
66 Spoto, M., Riera Puma, J.P., Fleming, E., Guan, C., Nzutchi, Y.O., Kim, D. et al. (2022) Large-scale CRISPRi and transcriptomics of *Staphylococcus epidermidis* identify genetic factors implicated in lifestyle versatility. *mBio* **13**, e02632-22 https://doi.org/10.1128/mbio.02632-22
67 Rostain, W., Grebert, T., Vyhovskyi, D., Pizarro, P.T., Van Bellingen, G.T., Cui, L. et al. (2023) Cas9 off-target binding to the promoter of bacterial genes leads to silencing and toxicity. *Nucleic Acids Res.* **51**, 3485–3496 https://doi.org/10.1093/nar/gkad170
68 Wang, T., Guan, C., Guo, J., Liu, B., Wu, Y., Xie, Z. et al. (2018) Pooled CRISPR interference screening enables genome-scale functional genomics study in bacteria with superior performance. *Nat. Commun.* **9**, 2475 https://doi.org/10.1038/s41467-018-04899-x
69 Rousset, F., Cui, L., Siouve, E., Becavin, C., Depardieu, F. and Bikard, D. (2018) Genome-wide CRISPR-dCas9 screens in *E. coli* identify essential genes and phage host factors. *PLoS Genet.* **14**, e1007749 https://doi.org/10.1371/journal.pgen.1007749

Biochemical Journal (2024) **481** 839–864
https://doi.org/10.1042/BCJ20220062

70 Silvis, M.R., Rajendram, M., Shi, H., Osadnik, H., Gray, A.N., Cesar, S. et al. (2021) Morphological and transcriptional responses to CRISPRi knockdown of essential genes in *Escherichia coli*. *mBio* **12**, e02561-21 https://doi.org/10.1128/mBio.02561-21
71 Peters, J.M., Koo, B.M., Patino, R., Heussler, G.E., Hearne, C.C., Qu, J. et al. (2019) Enabling genetic analysis of diverse bacteria with mobile-CRISPRi. *Nat. Microbiol.* **4**, 244–250 https://doi.org/10.1038/s41564-018-0327-z
72 Hawkins, J.S., Silvis, M.R., Koo, B.M., Peters, J.M., Osadnik, H., Jost, M. et al. (2020) Mismatch-CRISPRi reveals the co-varying expression-fitness relationships of essential genes in *Escherichia coli* and *Bacillus subtilis*. *Cell Syst.* **11**, 523–535.e9 https://doi.org/10.1016/j.cels.2020.09.009
73 de Wet, T.J., Winkler, K.R., Mhlanga, M., Mizrahi, V. and Warner, D.F. (2020) Arrayed CRISPRi and quantitative imaging describe the morphotypic landscape of essential mycobacterial genes. *Elife* **9**, e60083 https://doi.org/10.7554/eLife.60083
74 Liu, X., Kimmey, J.M., Matarazzo, L., de Bakker, V., Van Maele, L., Sirard, J.C. et al. (2021) Exploration of bacterial bottlenecks and *Streptococcus pneumoniae* pathogenesis by CRISPRi-Seq. *Cell Host Microbe* **29**, 107–120.e6 https://doi.org/10.1016/j.chom.2020.10.001
75 Mathis, A.D., Otto, R.M. and Reynolds, K.A. (2021) A simplified strategy for titrating gene expression reveals new relationships between genotype, environment, and bacterial growth. *Nucleic Acids Res.* **49**, e6 https://doi.org/10.1093/nar/gkaa1073
76 Liu, Y., Wan, X. and Wang, B. (2019) Engineered CRISPRa enables programmable eukaryote-like gene activation in bacteria. *Nat. Commun.* **10**, 3693 https://doi.org/10.1038/s41467-019-11479-0
77 Dong, C., Fontana, J., Patel, A., Carothers, J.M. and Zalatan, J.G. (2018) Synthetic CRISPR-Cas gene activators for transcriptional reprogramming in bacteria. *Nat. Commun.* **9**, 2489 https://doi.org/10.1038/s41467-018-04901-6
78 Fontana, J., Dong, C., Kiattisewee, C., Chavali, V.P., Tickman, B.I., Carothers, J.M. et al. (2020) Effective CRISPRa-mediated control of gene expression in bacteria must overcome strict target site requirements. *Nat. Commun.* **11**, 1618 https://doi.org/10.1038/s41467-020-15454-y
79 Gilbert, L.A., Horlbeck, M.A., Adamson, B., Villalta, J.E., Chen, Y., Whitehead, E.H. et al. (2014) Genome-scale CRISPR-mediated control of gene repression and activation. *Cell* **159**, 647–661 https://doi.org/10.1016/j.cell.2014.09.029
80 Villegas Kcam, M.C., Tsong, A.J. and Chappell, J. (2021) Rational engineering of a modular bacterial CRISPR–Cas activation platform with expanded target range. *Nucleic Acids Res.* **49**, 4793–4802 https://doi.org/10.1093/nar/gkab211
81 Kirstein, J., Hoffmann, A., Lilie, H., Schmidt, R., Helga, R.W., Heike, B.O. et al. (2009) The antibiotic ADEP reprogrammes ClpP, switching it from a regulated to an uncontrolled protease. *EMBO Mol. Med.* **1**, 37–49 https://doi.org/10.1002/emmm.200900002
82 Apfel, C.M., Takács, B., Fountoulakis, M., Stieger, M. and Keck, W. (1999) Use of genomics to identify bacterial undecaprenyl pyrophosphate synthetase: cloning, expression, and characterization of the essential uppS gene. *J. Bacteriol.* **181**, 483–492 https://doi.org/10.1128/JB.181.2.483-492.1999
83 Good, L. and Nielsen, P.E. (1998) Antisense inhibition of gene expression in bacteria by PNA targeted to mRNA. *Nat. Biotechnol.* **16**, 355–358 https://doi.org/10.1038/nbt0498-355
84 Kernodle, D.S., Voladri, R.K.R., Menzies, B.E., Hager, C.C. and Edwards, K.M. (1997) Expression of an antisense hla fragment in *Staphylococcus aureus* reduces alpha-toxin production in vitro and attenuates lethal activity in a murine model. *Infect. Immun.* **65**, 179–184 https://doi.org/10.1128/iai.65.1.179-184.1997
85 Meng, J., Kanzaki, G., Meas, D., Lam, C.K., Crummer, H., Tain, J. et al. (2012) A genome-wide inducible phenotypic screen identifies antisense RNA constructs silencing *Escherichia coli* essential genes. *FEMS Microbiol. Lett.* **329**, 45–53 https://doi.org/10.1111/j.1574-6968.2012.02503.x
86 Forsyth, R.A., Haselbeck, R.J., Ohlsen, K.L., Yamamoto, R.T., Xu, H., Trawick, J.D. et al. (2002) A genome-wide strategy for the identification of essential genes in *Staphylococcus aureus*. *Mol. Microbiol.* **43**, 1387–1400 https://doi.org/10.1046/j.1365-2958.2002.02832.x
87 Ji, Y., Zhang, B., Van Horn, S.F., Warren, P., Woodnutt, G., Burnham, M.K.R. et al. (2001) Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA. *Science* **293**, 2266–2269 https://doi.org/10.1126/science.1063566
88 Rusmini, R., Vecchietti, D., Macchi, R., Vidal-Aroca, F. and Bertoni, G. (2014) A shotgun antisense approach to the identification of novel essential genes in *Pseudomonas aeruginosa*. *BMC Microbiol.* **14**, 24 https://doi.org/10.1186/1471-2180-14-24
89 Traykovska, M. and Penchovsky, R. (2022) Engineering antisense oligonucleotides as antibacterial agents that target FMN riboswitches and inhibit the growth of *Staphylococcus aureus*, *Listeria monocytogenes*, and *Escherichia coli*. *ACS Synth. Biol.* **11**, 1845–1855 https://doi.org/10.1021/acssynbio.2c00013
90 Traykovska, M., Popova, K.B. and Penchovsky, R. (2021) Targeting glmS ribozyme with chimeric antisense oligonucleotides for antibacterial drug development. *ACS Synth. Biol.* **10**, 3167–3176 https://doi.org/10.1021/acssynbio.1c00443
91 McGinness, K.E., Baker, T.A. and Sauer, R.T. (2006) Engineering controllable protein degradation. *Mol. Cell* **22**, 701–707 https://doi.org/10.1016/j.molcel.2006.04.027
92 Griffith, K.L. and Grossman, A.D. (2008) Inducible protein degradation in *Bacillus subtilis* using heterologous peptide tags and adaptor proteins to target substrates to the protease ClpXP. *Mol. Microbiol.* **70**, 1012–1025 https://doi.org/10.1111/j.1365-2958.2008.06467.x
93 Kim, J.-H., Wei, J.-R., Wallach, J.B., Robbins, R.S., Rubin, E.J. and Schnappinger, D. (2011) Protein inactivation in mycobacteria by controlled proteolysis and its application to deplete the beta subunit of RNA polymerase. *Nucleic Acids Res.* **39**, 2210–2220 https://doi.org/10.1093/nar/gkq1149
94 Davis, J.H., Baker, T.A. and Sauer, R.T. (2011) Small-molecule control of protein degradation using split adaptors. *ACS Chem. Biol.* **6**, 1205–1213 https://doi.org/10.1021/cb2001389
95 Durante-Rodríguez, G., De Lorenzo, V. and Nikel, P.I. (2018) A post-translational metabolic switch enables complete decoupling of bacterial growth from biopolymer production in engineered *Escherichia coli*. *ACS Synth. Biol.* **7**, 2686–2697 https://doi.org/10.1021/acssynbio.8b00345
96 Cameron, D.E. and Collins, J.J. (2014) Tunable protein degradation in bacteria. *Nat. Biotechnol.* **32**, 1276–1281 https://doi.org/10.1038/nbt.3053
97 Morreale, F.E., Kleine, S., Leodolter, J., Junker, S., Hoi, D.M., Ovchinnikov, S. et al. (2022) BacPROTACs mediate targeted protein degradation in bacteria. *Cell* **185**, 2338–2353.e18 https://doi.org/10.1016/j.cell.2022.05.009
98 Palmer, A.C. and Kishony, R. (2014) Opposing effects of target overexpression reveal drug mechanisms. *Nat. Commun.* **5**, 4269 https://doi.org/10.1038/ncomms5296
99 Chakraborty, S., Gruber, T., Barry, C.E., Boshoff, H.I. and Rhee, K.Y. (2013) Para-aminosalicylic acid acts as an alternative substrate of folate metabolism in *Mycobacterium tuberculosis*. *Science* **339**, 88–91 https://doi.org/10.1126/science.1228980
100 Wei, J.R., Krishnamoorthy, V., Murphy, K., Kim, J.H., Schnappinger, D., Alber, T. et al. (2011) Depletion of antibiotic targets has widely varying effects on growth. *Proc. Natl Acad. Sci. U.S.A.* **108**, 4176–4181 https://doi.org/10.1073/pnas.1018301108

101 Fell, D.A. (1992) Metabolic control analysis: a survey of its theoretical and experimental development. *Biochem. J.* **286**, 313–330 https://doi.org/10.1042/bj2860313
102 Costanzo, M., VanderSluis, B., Koch, E.N., Baryshnikova, A., Pons, C., Tan, G. et al. (2016) A global genetic interaction network maps a wiring diagram of cellular function. *Science* **353**, aaf1420 https://doi.org/10.1126/science.aaf1420
103 Costanzo, M., Hou, J., Messier, V., Nelson, J., Rahman, M., VanderSluis, B. et al. (2021) Environmental robustness of the global yeast genetic interaction network. *Science* **372**, eabf8424 https://doi.org/10.1126/science.abf8424
104 Butland, G., Babu, M., Díaz-Mejía, J.J., Bohdana, F., Phanse, S., Gold, B. et al. (2008) eSGA: *E. coli* synthetic genetic array analysis. *Nat. Methods* **5**, 789–795 https://doi.org/10.1038/nmeth.1239
105 Typas, A., Nichols, R.J., Siegele, D.A., Shales, M., Collins, S.R., Lim, B. et al. (2008) High-throughput, quantitative analyses of genetic interactions in *E. coli*. *Nat. Methods* **5**, 781–787 https://doi.org/10.1038/nmeth.1240
106 Mutalik, V.K., Novichkov, P.S., Price, M.N., Owens, T.K., Callaghan, M., Carim, S. et al. (2019) Dual-barcoded shotgun expression library sequencing for high-throughput characterization of functional traits in bacteria. *Nat. Commun.* **10**, 308 https://doi.org/10.1038/s41467-018-08177-8
107 Warner, J.R., Reeder, P.J., Karimpour-Fard, A., Woodruff, L.B.A. and Gill, R.T. (2010) Rapid profiling of a microbial genome using mixtures of barcoded oligonucleotides. *Nat. Biotechnol.* **28**, 856–862 https://doi.org/10.1038/nbt.1653
108 Freed, E.F., Winkler, J.D., Weiss, S.J., Garst, A.D., Mutalik, V.K., Arkin, A.P. et al. (2015) Genome-wide tuning of protein expression levels to rapidly engineer microbial traits. *ACS Synth. Biol.* **4**, 1244–1253 https://doi.org/10.1021/acssynbio.5b00133
109 Cheng, A.A., Ding, H. and Lu, T.K. (2014) Enhanced killing of antibiotic-resistant bacteria enabled by massively parallel combinatorial genetics. *Proc. Natl Acad. Sci. U.S.A.* **111**, 12462–12467 https://doi.org/10.1073/pnas.1400093111
110 McNeil, M.B., Keighley, L.M., Cook, J.R., Cheung, C.Y. and Cook, G.M. (2021) CRISPR interference identifies vulnerable cellular pathways with bactericidal phenotypes in *Mycobacterium tuberculosis*. *Mol. Microbiol.* **116**, 1033–1043 https://doi.org/10.1111/mmi.14790
111 Otoupal, P.B., Eller, K.A., Erickson, K.E., Campos, J., Aunins, T.R. and Chatterjee, A. (2021) Potentiating antibiotic efficacy via perturbation of non-essential gene expression. *Commun. Biol.* **4**, 1267 https://doi.org/10.1038/s42003-021-02783-x
112 Ellis, N.A., Myers, K.S., Tung, J., Davidson Ward, A., Johnston, K., Bonnington, K.E. et al. (2023) A randomized multiplex CRISPRi-Seq approach for the identification of critical combinations of genes. *Elife* **12**, RP86903 https://doi.org/10.7554/eLife.86903
113 Rachwalski, K., Tu, M.M., Madden, S.J., French, S., Hansen, D.M. and Brown, E.D. (2024) A mobile CRISPRi collection enables genetic interaction studies for the essential genes of *Escherichia coli*. *Cell Rep. Methods* **4**, 100693 https://doi.org/10.1016/j.crmeth.2023.100693
114 Ranava, D., Yang, Y., Orenday-Tapia, L., Rousset, F., Turlan, C., Morales, V. et al. (2021) Lipoprotein DolP supports proper folding of BamA in the bacterial outer membrane promoting fitness upon envelope stress. *Elife* **10**, e67817 https://doi.org/10.7554/eLife.67817
115 Otto, R.M., Turska-Nowak, A., Brown, P.M. and Reynolds, K.A. (2024) A continuous epistasis model for predicting growth rate given combinatorial variation in gene expression and environment. *Cell Syst.* **15**, 134–148.e7 https://doi.org/10.1016/j.cels.2024.01.003
116 Giaever, G., Shoemaker, D.D., Jones, T.W., Liang, H., Winzeler, E.A., Astromoff, A. et al. (1999) Genomic profiling of drug sensitivities via induced haploinsufficiency. *Nat. Genet.* **21**, 278–283 https://doi.org/10.1038/6791
117 Nichols, R.J., Sen, S., Choo, Y.J., Beltrao, P., Zietek, M., Chaba, R. et al. (2011) Phenotypic landscape of a bacterial cell. *Cell* **144**, 143–156 https://doi.org/10.1016/j.cell.2010.11.052
118 Tamae, C., Liu, A., Kim, K., Sitz, D., Hong, J., Becket, E. et al. (2008) Determination of antibiotic hypersensitivity among 4,000 single-gene-knockout mutants of *Escherichia coli*. *J. Bacteriol.* **190**, 5981–5988 https://doi.org/10.1128/JB.01982-07
119 Hogan, A.M., Rahman, A.S.M.Z., Motnenko, A., Natarajan, A., Maydaniuk, D.T., León, B. et al. (2023) Profiling cell envelope-antibiotic interactions reveals vulnerabilities to β-lactams in a multidrug-resistant bacterium. *Nat. Commun.* **14**, 4815 https://doi.org/10.1038/s41467-023-40494-5
120 Girgis, H.S., Hottes, A.K. and Tavazoie, S. (2009) Genetic architecture of intrinsic antibiotic susceptibility. *PLoS One* **4**, e5629 https://doi.org/10.1371/journal.pone.0005629
121 Liu, A., Tran, L., Becket, E., Lee, K., Chinn, L., Park, E. et al. (2010) Antibiotic sensitivity profiles determined with an *Escherichia coli* gene knockout collection: generating an antibiotic bar code. *Antimicrob. Agents Chemother.* **54**, 1393–1403 https://doi.org/10.1128/AAC.00906-09
122 Jana, B., Cain, A.K., Doerrler, W.T., Boinett, C.J., Fookes, M.C., Parkhill, J. et al. (2017) The secondary resistome of multidrug-resistant *Klebsiella pneumoniae*. *Sci. Rep.* **7**, 42483 https://doi.org/10.1038/srep42483
123 French, S., Mangat, C., Bharat, A., Cté, J.P., Mori, H. and Brown, E.D. (2016) A robust platform for chemical genomics in bacterial systems. *Mol. Biol. Cell* **27**, 1015–1025 https://doi.org/10.1091/mbc.E15-08-0573
124 Price, M.N., Wetmore, K.M., Waters, R.J., Callaghan, M., Ray, J., Liu, H. et al. (2018) Mutant phenotypes for thousands of bacterial genes of unknown function. *Nature* **557**, 503–509 https://doi.org/10.1038/s41586-018-0124-0
125 Fajardo, A., Martínez-Martín, N., Mercadillo, M., Galán, J.C., Ghysels, B., Matthijs, S. et al. (2008) The neglected intrinsic resistome of bacterial pathogens. *PLoS One* **3**, e1619 https://doi.org/10.1371/journal.pone.0001619
126 Singh, S.B., Zink, D.L., Dorso, K., Motyl, M., Salazar, O., Basilio, A. et al. (2009) Isolation, structure, and antibacterial activities of lucensimycins D-G, discovered from streptomyces lucensis MA7349 using an antisense strategy. *J. Nat. Prod.* **72**, 345–352 https://doi.org/10.1021/np8005106
127 Young, K., Jayasuriya, H., Ondeyka, J.G., Herath, K., Zhang, C., Kodali, S. et al. (2006) Discovery of FabH/FabF inhibitors from natural products. *Antimicrob. Agents Chemother.* **50**, 519–526 https://doi.org/10.1128/AAC.50.2.519-526.2006
128 Wang, J., Soisson, S.M., Young, K., Shoop, W., Kodali, S., Galgoci, A. et al. (2006) Platensimycin is a selective FabF inhibitor with potent antibiotic properties. *Nature* **441**, 358–361 https://doi.org/10.1038/nature04784
129 Johnson, E.O., LaVerriere, E., Office, E., Stanley, M., Meyer, E., Kawate, T. et al. (2019) Large-scale chemical–genetics yields new *M. tuberculosis* inhibitor classes. *Nature* **571**, 72–78 https://doi.org/10.1038/s41586-019-1315-z
130 Anglada-Girotto, M., Handschin, G., Ortmayr, K., Campos, A.I., Gillet, L., Manfredi, P. et al. (2022) Combining CRISPRi and metabolomics for functional annotation of compound libraries. *Nat. Chem. Biol.* **18**, 482–491 https://doi.org/10.1038/s41589-022-00970-3
131 Li, S., Poulton, N.C., Chang, J.S., Azadian, Z.A., DeJesus, M.A., Ruecker, N. et al. (2022) CRISPRi chemical genetics and comparative genomics identify genes mediating drug potency in *Mycobacterium tuberculosis*. *Nat. Microbiol.* **7**, 766–779 https://doi.org/10.1038/s41564-022-01130-y
132 Vilchèze, C., Av-Gay, Y., Barnes, S.W., Larsen, M.H., Walker, J.R., Glynne, R.J. et al. (2011) Coresistance to isoniazid and ethionamide maps to mycothiol biosynthetic genes in *Mycobacterium bovis*. *Antimicrob. Agents Chemother.* **55**, 4422–4423 https://doi.org/10.1128/AAC.00564-11

Biochemical Journal (2024) **481** 839–864
https://doi.org/10.1042/BCJ20220062

133 Moradigaravand, D., Grandjean, L., Martinez, E., Li, H., Zheng, J., Coronel, J. et al. (2016) *dfra* thyA double deletion in *para*-Aminosalicylic acid-resistant *Mycobacterium tuberculosis* Beijing strains. *Antimicrob. Agents Chemother.* **60**, 3864–3867 https://doi.org/10.1128/AAC.00253-16
134 D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C. et al. (2011) Antibiotic resistance is ancient. *Nature* **477**, 457–461 https://doi.org/10.1038/nature10388
135 Waglechner, N., McArthur, A.G. and Wright, G.D. (2019) Phylogenetic reconciliation reveals the natural history of glycopeptide antibiotic biosynthesis and resistance. *Nat. Microbiol.* **4**, 1862–1871 https://doi.org/10.1038/s41564-019-0531-5
136 Hall, B.G. and Barlow, M. (2004) Evolution of the serine β-lactamases: past, present and future. *Drug Resist. Updat.* **7**, 111–123 https://doi.org/10.1016/j.drup.2004.02.003
137 Barlow, M. and Hall, B.G. (2002) Phylogenetic analysis shows that the OXA β-lactamase genes have been on plasmids for millions of years. *J. Mol. Evol.* **55**, 314–321 https://doi.org/10.1007/s00239-002-2328-y
138 Firn, R.D. and Jones, C.G. (2003) Natural products – a simple model to explain chemical diversity. *Nat. Prod. Rep.* **20**, 382–391 https://doi.org/10.1039/b208815k
139 Wright, G.D. (2007) The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat. Rev. Microbiol.* **5**, 175–186 https://doi.org/10.1038/nrmicro1614
140 Almabruk, K.H., Dinh, L.K. and Philmus, B. (2018) Self-resistance of natural product producers: past, present, and future focusing on self-resistant protein variants. *ACS Chem. Biol.* **13**, 1426–1437 https://doi.org/10.1021/acschembio.8b00173
141 Mak, S., Xu, Y. and Nodwell, J.R. (2014) The expression of antibiotic resistance genes in antibiotic-producing bacteria. *Mol. Microbiol.* **93**, 391–402 https://doi.org/10.1111/mmi.12689
142 D'Costa, V.M., McGrann, K.M., Hughes, D.W. and Wright, G.D. (2006) Sampling the antibiotic resistome. *Science* **311**, 374–377 https://doi.org/10.1126/science.1120800
143 Riesenfeld, S., Goodman, R.M. and Handelsman, J. (2004) Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environ. Microbiol.* **6**, 981–989 https://doi.org/10.1111/j.1462-2920.2004.00664.x
144 Palace, S.G., Wang, Y., Rubin, D.H.F., Welsh, M.A., Mortimer, T.D., Cole, K. et al. (2020) RNA polymerase mutations cause cephalosporin resistance in clinical *Neisseria gonorrhoeae* isolates. *Elife* **9**, e51407 https://doi.org/10.7554/eLife.51407
145 Silver, L.L. and Bostian, K.A. (1993) Discovery and development of new antibiotics: the problem of antibiotic resistance. *Antimicrob. Agents Chemother.* **37**, 377–383 https://doi.org/10.1128/AAC.37.3.377
146 Silver, L.L. (2007) Multi-targeting by monotherapeutic antibacterials. *Nat. Rev. Drug Discov.* **6**, 41–55 https://doi.org/10.1038/nrd2202
147 Darby, E.M., Trampari, E., Siasat, P., Gaya, M.S., Alav, I., Webber, M.A. et al. (2023) Molecular mechanisms of antibiotic resistance revisited. *Nat. Rev. Microbiol.* **21**, 280–295 https://doi.org/10.1038/s41579-022-00820-y
148 Hon, W.C., McKay, G.A., Thompson, P.R., Sweet, R.M., Yang, D.S.C., Wright, G.D. et al. (1997) Structure of an enzyme required for aminoglycoside antibiotic resistance reveals homology to eukaryotic protein kinases. *Cell* **89**, 887–895 https://doi.org/10.1016/s0092-8674(00)80274-3
149 Traub, B. and Beck, C.F. (1985) Resistance to various tetracyclines mediated by transposon Tn10 in *Escherichia coli* K-12. *Antimicrob. Agents Chemother.* **27**, 879–881 https://doi.org/10.1128/AAC.27.5.879
150 Piepersberg, W., Distler, J., Heinzel, P. and Perez-Gonzalez, J.-A. (1988) Antibiotic resistance by modification: many resistance genes could be derived from cellular control genes in actinomycetes - a hypothesis. *Actinomycetologica* **2**, 83–98 https://doi.org/10.3209/saj.2_83
151 Paul, S., Million-Weaver, S., Chattopadhyay, S., Sokurenko, E. and Merrikh, H. (2013) Accelerated gene evolution through replication–transcription conflicts. *Nature* **495**, 512–515 https://doi.org/10.1038/nature11989
152 Johnson, E.O., Office, E., Kawate, T., Orzechowski, M. and Hung, D.T. (2020) Large-scale chemical-genetic strategy enables the design of antimicrobial combination chemotherapy in mycobacteria. *ACS Infect. Dis.* **6**, 56–63 https://doi.org/10.1021/acsinfecdis.9b00373
153 Rubin, D.H.F., Ma, K.C., Westervelt, K.A., Hullahalli, K., Waldor, M.K. and Grad, Y.H. (2023) Canb is a metabolic mediator of antibiotic resistance in *Neisseria gonorrhoeae*. *Nat. Microbiol.* **8**, 28–39 https://doi.org/10.1038/s41564-022-01282-x
154 Hochberg, G.K.A., Liu, Y., Marklund, E.G. Metzger, B.P.H., Laganowsky, A. and Thornton, J.W. (2020) A hydrophobic ratchet entrenches molecular complexes. *Nature* **588**, 503–508 https://doi.org/10.1038/s41586-020-3021-2
155 Schober, A.F., Mathis, A.D., Ingle, C., Park, J.O., Chen, L., Rabinowitz, J.D. et al. (2019) A two-enzyme adaptive unit within bacterial folate metabolism. *Cell Rep.* **27**, 3359–3370.e7 https://doi.org/10.1016/j.celrep.2019.05.030
156 Podgornaia, A.I. and Laub, M.T. (2015) Pervasive degeneracy and epistasis in a protein-protein interface. *Science* **347**, 673–677 https://doi.org/10.1126/science.1257360
157 Peregrín-Alvarez, J.M., Sanford, C. and Parkinson, J. (2009) The conservation and evolutionary modularity of metabolism. *Genome Biol.* **10**, R63 https://doi.org/10.1186/gb-2009-10-6-r63
158 Eagle, H. and Musselman, A.D. (1949) The slow recovery of bacteria from the toxic effects of penicillin. *J. Bacteriol.* **58**, 475–490 https://doi.org/10.1128/jb.58.4.475-490.1949
159 Wiesch, A.Z., Abel, P., Gkotzis, S., Ocampo, S., Engelstädter, P., Hinkley, J. (2015) Classic reaction kinetics can explain complex patterns of antibiotic action. *Sci. Transl. Med.* **7**, 287ra73 https://doi.org/10.1126/scitranslmed.aaa8760
160 Mackenzie, F.M. and Gould, I.M. (1993) The post-antibiotic effect. *J. Antimicrob. Chemother.* **32**, 519–537 https://doi.org/10.1093/jac/32.4.519
161 Stubbings, W., Bostock, J., Ingham, E. and Chopra, I. (2005) Deletion of the multiple-drug efflux pump AcrAB in *Escherichia coli* prolongs the postantibiotic effect. *Antimicrob. Agents Chemother.* **49**, 1206–1208 https://doi.org/10.1128/AAC.49.3.1206-1208.2005
162 Kaplan, Y., Reich, S., Oster, E., Maoz, S., Levin-Reisman, I., Ronin, I. et al. (2021) Observation of universal ageing dynamics in antibiotic persistence. *Nature* **600**, 290–294 https://doi.org/10.1038/s41586-021-04114-w
163 Prideaux, B., Via, L.E., Zimmerman, M.D., Eum, S., Sarathy, J., O'Brien, P. et al. (2015) The association between sterilizing activity and drug distribution into tuberculosis lesions. *Nat. Med.* **21**, 1223–1227 https://doi.org/10.1038/nm.3937
164 Gallagher, L.A., Bailey, J. and Manoil, C. (2020) Ranking essential bacterial processes by speed of mutant death. *Proc. Natl Acad. Sci. U.S.A.* **117**, 18010–18017 https://doi.org/10.1073/pnas.2001507117
165 Tettelin, H., Masignani, V., Cieslewicz, M.J., Donati, C., Medini, D., Ward, N.L. et al. (2005) Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial 'pan-genome'. *Proc. Natl Acad. Sci. U.S.A.* **102**, 13950–13955 https://doi.org/10.1073/pnas.0506758102

166 Ghomi, F.A., Langridge, G.C., Cain, A.K., Boinett, C., Abd, M., Ghany, E. et al. (2022) High-throughput transposon mutagenesis in the family Enterobacteriaceae reveals core essential genes and rapid turnover of essentiality. *bioRxiv* https://doi.org/10.1101/2022.10.20.512852
167 Rosconi, F., Rudmann, E., Li, J., Surujon, D., Anthony, J., Frank, M. et al. (2022) A bacterial pan-genome makes gene essentiality strain-dependent and evolvable. *Nat. Microbiol.* **7**, 1580–1592 https://doi.org/10.1038/s41564-022-01208-7
168 Gentry, D.R., Ingraham, K.A., Stanhope, M.J., Rittenhouse, S., Jarvest, R.L., O'Hanlon, P.J. et al. (2003) Variable sensitivity to bacterial methionyl-tRNA synthetase inhibitors reveals subpopulations of *Streptococcus pneumoniae* with two distinct methionyl-tRNA synthetase genes. *Antimicrob. Agents Chemother.* **47**, 1784–1789 https://doi.org/10.1128/AAC.47.6.1784-1789.2003
169 Carey, A.F., Rock, J.M., Krieger, I.V., Chase, M.R., Fernandez-Suarez, M., Gagneux, S. et al. (2018) Tnseq of *Mycobacterium tuberculosis* clinical isolates reveals strain-specific antibiotic liabilities. *PLoS Pathog.* **14**, e1006939 https://doi.org/10.1371/journal.ppat.1006939
170 Hensel, M., Shea, J., Gleeson, C., Jones, M.D., Dalton, E. and Holden, D.W. (1995) Simultaneous identification of bacterial virulence genes by negative selection. *Science* **269**, 400–403 https://doi.org/10.1126/science.7618105
171 Umland, T.C., Wayne Schultz, L., MacDonald, U., Beanan, J.M., Olson, R. and Russo, T.A. (2012) In vivo-validated essential genes identified in *Acinetobacter baumannii* by using human ascites overlap poorly with essential genes detected on laboratory media. *mBio* **3**, e00113-12 https://doi.org/10.1128/mBio.00113-12
172 Pethe, K., Sequeira, P.C., Agarwalla, S., Rhee, K., Kuhen, K., Phong, W.Y. et al. (2010) A chemical genetic screen in *Mycobacterium tuberculosis* identifies carbon-source-dependent growth inhibitors devoid of in vivo efficacy. *Nat. Commun.* **1**, 57 https://doi.org/10.1038/ncomms1060
173 Brinster, S., Lamberet, G., Staels, B., Trieu-Cuot, P., Gruss, A. and Poyart, C. (2009) Type II fatty acid synthesis is not a suitable antibiotic target for Gram-positive pathogens. *Nature* **458**, 83–86 https://doi.org/10.1038/nature07772
174 Ibberson, C.B., Stacy, A., Fleming, D., Dees, J.L., Rumbaugh, K., Gilmore, M.S. et al. (2017) Co-infecting microorganisms dramatically alter pathogen gene essentiality during polymicrobial infection. *Nat. Microbiol.* **2**, 17079 https://doi.org/10.1038/nmicrobiol.2017.79
175 Sadybekov, A.A., Sadybekov, A.V., Liu, Y., Iliopoulos-Tsoutsouvas, C., Huang, X.P., Pickett, J. et al. (2022) Synthon-based ligand discovery in virtual libraries of over 11 billion compounds. *Nature* **601**, 452–459 https://doi.org/10.1038/s41586-021-04220-9
176 Ling, L.L., Schneider, T., Peoples, A.J., Spoering, A.L., Engels, I., Conlon, B.P. et al. (2015) A new antibiotic kills pathogens without detectable resistance. *Nature* **517**, 455–459 https://doi.org/10.1038/nature14098
177 Shukla, R., Peoples, A.J., Ludwig, K.C., Maity, S., Derks, M.G.N., De Benedetti, S. et al. (2023) An antibiotic from an uncultured bacterium binds to an immutable target. *Cell* **186**, 4059–4073.e27 https://doi.org/10.1016/j.cell.2023.07.038
178 Imai, Y., Meyer, K.J., Iinishi, A., Favre-Godal, Q., Green, R., Manuse, S. et al. (2019) A new antibiotic selectively kills Gram-negative pathogens. *Nature* **576**, 459–464 https://doi.org/10.1038/s41586-019-1791-1
179 Covington, B.C., Xu, F. and Seyedsayamdost, M.R. (2021) A natural product chemist's guide to unlocking silent biosynthetic gene clusters. *Annu. Rev. Biochem.* **90**, 763–788 https://doi.org/10.1146/annurev-biochem-081420-102432
180 Hopwood, D.A., Malpartida, F., Kieser, H.M., Ikeda, H., Duncan, J., Fujii, I. et al. (1985) Production of 'hybrid' antibiotics by genetic engineering. *Nature* **314**, 642–644 https://doi.org/10.1038/314642a0
181 Kim, E., Moore, B.S. and Yoon, Y.J. (2015) Reinvigorating natural product combinatorial biosynthesis with synthetic biology. *Nat. Chem. Biol.* **11**, 649–659 https://doi.org/10.1038/nchembio.1893
182 Ren, H., Shi, C. and Zhao, H. (2020) Computational tools for discovering and engineering natural product biosynthetic pathways. *iScience* **23**, 100795 https://doi.org/10.1016/j.isci.2019.100795
183 Medema, M.H., Blin, K., Cimermancic, P., De Jager, V., Zakrzewski, P., Fischbach, M.A. et al. (2011) antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. *Nucleic Acids Res.* **39**, W339–W346 https://doi.org/10.1093/nar/gkr466
184 Blin, K., Pascal Andreu, V., De Los Santos, E.L.C., Del Carratore, F., Lee, S.Y., Medema, M.H. et al. (2019) The antiSMASH database version 2: a comprehensive resource on secondary metabolite biosynthetic gene clusters. *Nucleic Acids Res.* **47**, D625–D630 https://doi.org/10.1093/nar/gky1060
185 Vila-Farres, X., Chu, J., Inoyama, D., Ternei, M.A., Lemetre, C., Cohen, L.J. et al. (2017) Antimicrobials inspired by nonribosomal peptide synthetase gene clusters. *J. Am. Chem. Soc.* **139**, 1404–1407 https://doi.org/10.1021/jacs.6b11861
186 Stansly, P.G. (1946) The presumptive identification of antibiotics. *Science* **103**, 402–403 https://doi.org/10.1126/science.103.2674.402.c
187 Peck, R.L., Hoffhixe, C.E. and Folker, K. (1946) Streptomyces antibiotics. IX. Dihydrostreptomycin. *J. Am. Chem. Soc.* **68**, 1390–1391 https://doi.org/10.1021/ja01211a513
188 Bartz, Q.R., Controulis, J., Crooks, H.M. and Rebstock, M.C. (1946) Dihydrostreptomycin. *J. Am. Chem. Soc.* **68**, 2163–2166 https://doi.org/10.1021/ja01215a013
189 Acred, P., Brown, D.M., Turner, D.H. and Wilson, M.J. (1962) Pharmacology and chemotherapy of ampicillin - a new broad-spectrum penicillin. *Br. J. Pharmacol. Chemother.* **18**, 356–369 https://doi.org/10.1111/j.1476-5381.1962.tb01416.x
190 Zampaloni, C., Mattei, P., Bleicher, K., Winther, L., Thäte, C., Bucher, C. et al. (2024) A novel antibiotic class targeting the lipopolysaccharide transporter. *Nature* **625**, 566–571 https://doi.org/10.1038/s41586-023-06873-0
191 Culp, E.J., Waglechner, N., Wang, W., Fiebig-Comyn, A.A., Hsu, Y.P., Koteva, K. et al. (2020) Evolution-guided discovery of antibiotics that inhibit peptidoglycan remodelling. *Nature* **578**, 582–587 https://doi.org/10.1038/s41586-020-1990-9
192 Libardo, M.D., Cervantes, J.L., Salazar, J.C. and Angeles-Boza, A.M. (2014) Improved bioactivity of antimicrobial peptides by addition of amino-terminal copper and nickel (ATCUN) binding motifs. *ChemMedChem* **9**, 1892–1901 https://doi.org/10.1002/cmdc.201402033
193 Libardo, M.D.J., Nagella, S., Lugo, A., Pierce, S. and Angeles-Boza, A.M. (2015) Copper-binding tripeptide motif increases potency of the antimicrobial peptide anoplin via reactive oxygen species generation. *Biochem. Biophys. Res. Commun.* **456**, 446–451 https://doi.org/10.1016/j.bbrc.2014.11.104
194 Burger, R.M. (1998) Cleavage of nucleic acids by bleomycin. *Chem. Rev.* **98**, 1153–1169 https://doi.org/10.1021/cr960438a
195 Silver, L.L. (2016) A Gestalt approach to Gram-negative entry. *Bioorg. Med. Chem.* **24**, 6379–6389 https://doi.org/10.1016/j.bmc.2016.06.044
196 Suginaka, H., Blumberg, P.M. and Strominger, J.L. (1972) Multiple penicillin-binding components in *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, and *Escherichia coli*. *J. Biol. Chem.* **247**, 5279–5288 https://doi.org/10.1016/S0021-9258(20)81102-8
197 Manchester, J.I., Buurman, E.T., Bisacchi, G.S. and McLaughlin, R.E. (2012) Molecular determinants of AcrB-mediated bacterial efflux implications for drug discovery. *J. Med. Chem.* **55**, 2532–2537 https://doi.org/10.1021/jm201275d

Biochemical Journal (2024) **481** 839–864
https://doi.org/10.1042/BCJ20220062

198 Brown, D.G., May-Dracka, T.L., Gagnon, M.M. and Tommasi, R. (2014) Trends and exceptions of physical properties on antibacterial activity for gram-positive and gram-negative pathogens. *J. Med. Chem.* **57**, 10144–10161 https://doi.org/10.1021/jm501552x
199 Goldman, R.C. (2013) Why are membrane targets discovered by phenotypic screens and genome sequencing in *Mycobacterium tuberculosis*? *Tuberculosis* **93**, 569–588 https://doi.org/10.1016/j.tube.2013.09.003
200 O'Shea, R. and Moser, H.E. (2008) Physicochemical properties of antibacterial compounds: implications for drug discovery. *J. Med. Chem.* **51**, 2871–2878 https://doi.org/10.1021/jm700967e
201 Richter, M.F., Drown, B.S., Riley, A.P., Garcia, A., Shirai, T., Svec, R.L. et al. (2017) Predictive compound accumulation rules yield a broad-spectrum antibiotic. *Nature* **545**, 299–304 https://doi.org/10.1038/nature22308
202 Krishnamoorthy, G., Leus, I.V., Weeks, J.W., Wolloscheck, D., Rybenkov, V.V. and Zgurskaya, H.I. (2017) Synergy between active efflux and outer membrane diffusion defines rules of antibiotic permeation into gram-negative bacteria. *mBio* **8**, e01172-17 https://doi.org/10.1128/mBio.01172-17
203 Prochnow, H., Fetz, V., Hotop, S.K., García-Rivera, M.A., Heumann, A. and Brönstrup, M. (2019) Subcellular quantification of uptake in gram-negative bacteria. *Anal Chem* **91**, 1863–1872 https://doi.org/10.1021/acs.analchem.8b03586
204 Geddes, E.J., Gugger, M.K., Garcia, A., Chavez, M.G., Lee, M.R., Perlmutter, S.J. et al. (2023) Porin-independent accumulation in Pseudomonas enables antibiotic discovery. *Nature* **624**, 145–153 https://doi.org/10.1038/s41586-023-06760-8
205 Leus, I.V., Adamiak, J., Chandar, B., Bonifay, V., Zhao, S., Walker, S.S. et al. (2023) Functional diversity of gram-negative permeability barriers reflected in antibacterial activities and intracellular accumulation of antibiotics. *Antimicrob. Agents Chemother.* **67**, e0137722 https://doi.org/10.1128/aac.01377-22
206 Motika, S.E., Ulrich, R.J., Geddes, E.J., Lee, H.Y., Lau, G.W. and Hergenrother, P.J. (2020) Gram-negative antibiotic active through inhibition of an essential riboswitch. *J. Am. Chem. Soc.* **142**, 10856–10862 https://doi.org/10.1021/jacs.0c04427
207 Spangler, B., Dovala, D., Sawyer, W.S., Thompson, K.V., Six, D.A., Reck, F. et al. (2018) Molecular probes for the determination of subcellular compound exposure profiles in gram-negative bacteria. *ACS Infect. Dis.* **4**, 1355–1367 https://doi.org/10.1021/acsinfecdis.8b00093
208 Widya, M., Pasutti, W.D., Sachdeva, M., Simmons, R.L., Tamrakar, P., Krucker, T. et al. (2019) Development and optimization of a higher-throughput bacterial compound accumulation assay. *ACS Infect. Dis.* **5**, 394–405 https://doi.org/10.1021/acsinfecdis.8b00299
209 Davis, T.D., Gerry, C.J. and Tan, D.S. (2014) General platform for systematic quantitative evaluation of small-molecule permeability in bacteria. *ACS Chem. Biol.* **9**, 2535–2544 https://doi.org/10.1021/cb5003015
210 Cooper, C.J., Krishnamoorthy, G., Wolloscheck, D., Walker, J.K., Rybenkov, V.V., Parks, J.M. et al. (2018) Molecular properties that define the activities of antibiotics in *Escherichia coli* and *Pseudomonas aeruginosa*. *ACS Infect. Dis.* **4**, 1223–1234 https://doi.org/10.1021/acsinfecdis.8b00036
211 Ude, J., Tripathi, V., Buyck, J.M., Söderholm, S., Cunrath, O., Fanous, J. et al. (2021) Outer membrane permeability: antimicrobials and diverse nutrients bypass porins in *Pseudomonas aeruginosa*. *Proc. Natl Acad. Sci. U.S.A.* **118**, e2107644118 https://doi.org/10.1073/pnas.2107644118
212 Parker, E.N., Cain, B.N., Hajian, B., Ulrich, R.J., Geddes, E.J., Barkho, S. et al. (2022) An iterative approach guides discovery of the FabI inhibitor fabimycin, a late-stage antibiotic candidate with in vivo efficacy against drug-resistant gram-negative infections. *ACS Cent. Sci.* **8**, 1145–1158 https://doi.org/10.1021/acscentsci.2c00598
213 Blasco, B., Jang, S., Terauchi, H., Kobayashi, N., Suzuki, S., Akao, Y. et al. (2024) High-throughput screening of small-molecules libraries identified antibacterials against clinically relevant multidrug-resistant *A. baumannii* and *K. pneumoniae*. *EBioMedicine* **102**, 105073 https://doi.org/10.1016/j.ebiom.2024.105073
214 Luckey, M., Pollack, J.R., Wayne, R., Ames, B.N. and Neilands, J.B. (1972) Iron uptake in *Salmonella typhimurium*: utilization of exogenous siderochromes as iron carriers. *J. Bacteriol.* **111**, 731–738 https://doi.org/10.1128/jb.111.3.731-738.1972
215 Hartmann, A., Fiedler, H.-P. and Braun, V. (1979) Uptake and conversion of the antibiotic albomycin by *Escherichia coli* K-12. *Eur. J. Biochem.* **99**, 517–524 https://doi.org/10.1111/j.1432-1033.1979.tb13283.x
216 Ito, A., Nishikawa, T., Matsumoto, S., Yoshizawa, H., Sato, T., Nakamura, R. et al. (2016) Siderophore cephalosporin cefiderocol utilizes ferric iron transporter systems for antibacterial activity against *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **60**, 7396–7401 https://doi.org/10.1128/AAC.01405-16
217 Ghosh, M., Miller, P.A., Möllmann, U., Claypool, W.D., Schroeder, V.A., Wolter, W.R. et al. (2017) Targeted antibiotic delivery: selective siderophore conjugation with daptomycin confers potent activity against multidrug resistant *Acinetobacter baumannii* both in vitro and in vivo. *J. Med. Chem.* **60**, 4577–4583 https://doi.org/10.1021/acs.jmedchem.7b00102
218 Strydom, N., Gupta, S.V., Fox, W.S., Via, L.E., Bang, H., Lee, M. et al. (2019) Tuberculosis drugs' distribution and emergence of resistance in patient's lung lesions: a mechanistic model and tool for regimen and dose optimization. *PLoS Med.* **16**, e1002773 https://doi.org/10.1371/journal.pmed.1002773
219 Greenwood, D.J., Dos Santos, M.S., Huang, S., Russell, M.R.G., Collinson, L.M., MacRae, J.I. et al. (2019) Subcellular antibiotic visualization reveals a dynamic drug reservoir in infected macrophages. *Science* **364**, 1279–1282 https://doi.org/10.1126/science.aat9689
220 Falagas, M.E. and Kasiakou, S.K. (2005) Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin. Infect. Dis.* **40**, 1333–1341 https://doi.org/10.1086/429323
221 Gajadeera, C., Willby, M.J., Green, K.D., Shaul, P., Fridman, M., Garneau-Tsodikova, S. et al. (2015) Antimycobacterial activity of DNA intercalator inhibitors of *Mycobacterium tuberculosis* primase DnaG. *J. Antibiot. (Tokyo)* **68**, 153–157 https://doi.org/10.1038/ja.2014.131
222 Porter, J., Edwards, C. and Pickup, R.W. (1995) Rapid assessment of physiological status in *Escherichia coli* using fluorescent probes. *J. Appl. Bacteriol.* **79**, 399–408 https://doi.org/10.1111/j.1365-2672.1995.tb03154.x
223 Rozwarski, D.A., Grant, G.A., Barton, D.H.R., Jacobs, W.R. and Sacchettini, J.C. (1998) Modification of the NADH of the isoniazid target (InhA) from *Mycobacterium tuberculosis*. *Science* **279**, 98–102 https://doi.org/10.1126/science.279.5347.98
224 Edfeldt, F.N.B., Folmer, R.H.A. and Breeze, A.L. (2011) Fragment screening to predict druggability (ligandability) and lead discovery success. *Drug Discov. Today* **16**, 284–287 https://doi.org/10.1016/j.drudis.2011.02.002
225 Yocum, R.R., Rasmussen, J.R. and Strominger, J.L. (1980) The mechanism of action of penicillin. Penicillin acylates the active site of *Bacillus stearothermophilus* D-alanine carboxypeptidase. *J. Biol. Chem.* **255**, 3977–3986 https://doi.org/10.1016/S0021-9258(19)85621-1
226 Campbell, E.A., Korzheva, N., Mustaev, A., Murakami, K., Nair, S., Goldfarb, A. et al. (2001) Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell* **104**, 901–912 https://doi.org/10.1016/s0092-8674(01)00286-0

227 Leung, E., Datti, A., Cossette, M., Goodreid, J., McCaw, S.E., Mah, M. et al. (2011) Activators of cylindrical proteases as antimicrobials: identification and development of small molecule activators of ClpP protease. *Chem. Biol.* **18**, 1167–1178 https://doi.org/10.1016/j.chembiol.2011.07.023
228 Brötz-Oesterhelt, H., Beyer, D., Kroll, H.P., Endermann, R., Ladel, C., Schroeder, W. et al. (2005) Dysregulation of bacterial proteolytic machinery by a new class of antibiotics. *Nat. Med.* **11**, 1082–1087 https://doi.org/10.1038/nm1306
229 Cho, H., Choi, Y., Min, K., Son, J.B., Park, H., Lee, H.H. et al. (2020) Over-activation of a nonessential bacterial protease DegP as an antibiotic strategy. *Commun. Biol.* **3**, 547 https://doi.org/10.1038/s42003-020-01266-9
230 Olakanmi, O., Kesavalu, B., Pasula, R., Abdalla, M.Y., Schlesinger, L.S. and Britigan, B.E. (2013) Gallium nitrate is efficacious in murine models of tuberculosis and inhibits key bacterial Fe-dependent enzymes. *Antimicrob. Agents Chemother.* **57**, 6074–6080 https://doi.org/10.1128/AAC.01543-13
231 Kaneko, Y., Thoendel, M., Olakanmi, O., Britigan, B.E. and Singh, P.K. (2007) The transition metal gallium disrupts *Pseudomonas aeruginosa* iron metabolism and has antimicrobial and antibiofilm activity. *J. Clin. Invest.* **117**, 877–888 https://doi.org/10.1172/JCI30783
232 Jakeman, P. and Smith, W.C.S. (1994) Thalidomide in leprosy reaction. *Lancet* **343**, 432–433 https://doi.org/10.1016/s0140-6736(94)92686-7
233 Hu, Y., Helm, J.S., Chen, L., Ye, X.Y. and Walker, S. (2003) Ramoplanin inhibits bacterial transglycosylases by binding as a dimer to lipid II. *J. Am. Chem. Soc.* **125**, 8736–8737 https://doi.org/10.1021/ja035217i
234 Perkins, H.R. (1969) Specificity of combination between mucopeptide precursors and vancomycin or ristocetin. *Biochem. J.* **111**, 195–205 https://doi.org/10.1042/bj1110195
235 Swarts, B.M., Holsclaw, C.M., Jewett, J.C., Alber, M., Fox, D.M., Siegrist, M.S. et al. (2012) Probing the mycobacterial trehalome with bioorthogonal chemistry. *J. Am. Chem. Soc.* **134**, 16123–16126 https://doi.org/10.1021/ja3062419
236 Choi, J., Wagner, L.J.S., Timmermans, S.B.P.E., Malaker, S.A., Schumann, B., Gray, M.A. et al. (2019) Engineering orthogonal polypeptide GalNAc-transferase and UDP-sugar pairs. *J. Am. Chem. Soc.* **141**, 13442–13453 https://doi.org/10.1021/jacs.9b04695
237 Schumann, B., Malaker, S.A., Wisnovsky, S.P., Debets, M.F., Agbay, A.J., Fernandez, D. et al. (2020) Bump-and-hole engineering identifies specific substrates of glycosyltransferases in living cells. *Mol. Cell* **78**, 824–834.e15 https://doi.org/10.1016/j.molcel.2020.03.030
238 Baell, J.B. and Holloway, G.A. (2010) New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J. Med. Chem.* **53**, 2719–2740 https://doi.org/10.1021/jm901137j
239 Lipinski, C.A., Lombardo, F., Dominy, B.W. and Feeney, P.J. (1997) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* **23**, 3–25 https://doi.org/10.1016/S0169-409X(96)00423-1
240 Congreve, M., Carr, R., Murray, C. and Jhoti, H. (2003) A 'rule of three'; for fragment-based lead discovery? *Drug Discov. Today* **8**, 876–877 https://doi.org/10.1016/s1359-6446(03)02831-9
241 King, A.M., Zhang, Z., Glassey, E., Siuti, P., Clardy, J. and Voigt, C.A. (2023) Systematic mining of the human microbiome identifies antimicrobial peptides with diverse activity spectra. *Nat. Microbiol.* **8**, 2420–2434 https://doi.org/10.1038/s41564-023-01524-6
242 Stokes, J.M., Yang, K., Swanson, K., Jin, W., Cubillos-Ruiz, A., Donghia, N.M. et al. (2020) A deep learning approach to antibiotic discovery. *Cell* **180**, 688–702.e13 https://doi.org/10.1016/j.cell.2020.01.021
243 Liu, G., Catacutan, D.B., Rathod, K., Swanson, K., Jin, W., Mohammed, J.C. et al. (2023) Deep learning-guided discovery of an antibiotic targeting *Acinetobacter baumannii*. *Nat. Chem. Biol.* **19**, 1342–1350 https://doi.org/10.1038/s41589-023-01349-8
244 Wong, F., Zheng, E.J., Valeri, J.A., Donghia, N.M., Anahtar, M.N., Omori, S. et al. (2023) Discovery of a structural class of antibiotics with explainable deep learning. *Nature* **626**, 177–185 https://doi.org/10.1038/s41586-023-06887-8
245 Lyu, J., Wang, S., Balius, T.E., Singh, I., Levit, A., Moroz, Y.S. et al. (2019) Ultra-large library docking for discovering new chemotypes. *Nature* **566**, 224–229 https://doi.org/10.1038/s41586-019-0917-9
246 Graff, D.E., Shakhnovich, E.I. and Coley, C.W. (2021) Accelerating high-throughput virtual screening through molecular pool-based active learning. *Chem. Sci.* **12**, 7866–7881 https://doi.org/10.1039/d0sc06805e
247 Gorantla, R., Kubincová, A., Suutari, B., Cossins, B.P. and Mey, A.S.J.S. (2024) Benchmarking active learning protocols for ligand-binding affinity prediction. *J. Chem. Inf. Model.* **64**, 1955–1965 https://doi.org/10.1021/acs.jcim.4c00220
248 Rubinstein, E. and Keynan, Y. (2014) Vancomycin revisited - 60 years later. *Front. Public Health* **2**, 217 https://doi.org/10.3389/fpubh.2014.00217
249 Murray, B.E. (2000) Vancomycin-resistant enterococcal infections. *N.Engl. J. Med.* **342**, 710–721 https://doi.org/10.1056/NEJM200003093421007
250 Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N. and Balaban, N.Q. (2017) Antibiotic tolerance facilitates the evolution of resistance. *Science* **355**, 826–830 https://doi.org/10.1126/science.aaj2191
251 Gomez, J.E., Kaufmann-Malaga, B.B., Wivagg, C.N., Kim, P.B., Silvis, M.R., Renedo, N. et al. (2017) Ribosomal mutations promote the evolution of antibiotic resistance in a multidrug environment. *Elife* **6**, e20420 https://doi.org/10.7554/eLife.20420
252 Thompson, S., Zhang, Y., Ingle, C., Reynolds, K.A. and Kortemme, T. (2020) Altered expression of a quality control protease in *E. coli* reshapes the in vivo mutational landscape of a model enzyme. *Elife* **9**, e53476 https://doi.org/10.7554/eLife.53476
253 Yadon, A.N., Maharaj, K., Adamson, J.H., Lai, Y.P., Sacchettini, J.C., Ioerger, T.R. et al. (2017) A comprehensive characterization of PncA polymorphisms that confer resistance to pyrazinamide. *Nat. Commun.* **8**, 588 https://doi.org/10.1038/s41467-017-00721-2
254 Thangamani, S., Mohammad, H., Abushahba, M.F.N., Sobreira, T.J.P., Hedrick, V.E., Paul, L.N. et al. (2016) Antibacterial activity and mechanism of action of auranofin against multi-drug resistant bacterial pathogens. *Sci. Rep.* **6**, 22571 https://doi.org/10.1038/srep22571
255 Harbut, M.B., Vilchèze, C., Luo, X., Hensler, M.E., Guo, H., Yang, B. et al. (2015) Auranofin exerts broad-spectrum bactericidal activities by targeting thiol-redox homeostasis. *Proc. Natl Acad. Sci. U.S.A.* **112**, 4453–4458 https://doi.org/10.1073/pnas.1504022112
256 McOsker, C.C. and Fitzpatrick, P.M. (1994) Nitrofurantoin: mechanism of action and implications for resistance development in common uropathogens. *J. Antimicrob. Chemother.* **33**, 23–30 https://doi.org/10.1093/jac/33.suppl_a.23
257 Adolph, C., Cheung, C.-Y., McNeil, M.B., Jowsey, W.J., Williams, Z.C., Hards, K. et al. (2024) A dual-targeting succinate dehydrogenase and F1F0-ATP synthase inhibitor rapidly sterilizes replicating and non-replicating *Mycobacterium tuberculosis*. *Cell Chem. Biol.* **31**, 683–698.e7 https://doi.org/10.1016/j.chembiol.2023.12.002
258 Khodursky, A.B., Zechiedrich, E.L. and Cozzarelli, N.R. (1995) Topoisomerase IV is a target of quinolones in *Escherichia coli*. *Proc. Natl Acad. Sci. U.S.A.* **92**, 11801–11805 https://doi.org/10.1073/pnas.92.25.11801
259 Wright, G.D. (2016) Antibiotic adjuvants: rescuing antibiotics from resistance. *Trends Microbiol.* **24**, 862–871 https://doi.org/10.1016/j.tim.2016.06.009

Biochemical Journal (2024) **481** 839–864
https://doi.org/10.1042/BCJ20220062

260 Dhanda, G., Acharya, Y. and Haldar, J. (2023) Antibiotic adjuvants: a versatile approach to combat antibiotic resistance. *ACS Omega* **8**, 10757–10783 https://doi.org/10.1021/acsomega.3c00312

261 Tyers, M. and Wright, G.D. (2019) Drug combinations: a strategy to extend the life of antibiotics in the 21st century. *Nat. Rev. Microbiol.* **17**, 141–155 https://doi.org/10.1038/s41579-018-0141-x

262 Reading, C. and Cole, M. (1977) Clavulanic acid: a beta lactamase inhibiting beta lactam from *Streptomyces clavuligerus*. *Antimicrob. Agents Chemother.* **11**, 852–857 https://doi.org/10.1128/AAC.11.5.852

263 Kaatz, G.W. and Seo, S.M. (1995) Inducible NorA-mediated multidrug resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **39**, 2650–2655 https://doi.org/10.1128/AAC.39.12.2650

264 Nelson, M.L. and Levy, S.B. (1999) Reversal of tetracycline resistance mediated by different bacterial tetracycline resistance determinants by an inhibitor of the Tet(B) antiport protein. *Antimicrob. Agents Chemother.* **43**, 1719–1724 https://doi.org/10.1128/AAC.43.7.1719

265 Renau, T.E., Léger, R., Flamme, E.M., Sangalang, J., She, M.W., Yen, R. et al. (1999) Inhibitors of efflux pumps in *Pseudomonas aeruginosa* potentiate the activity of the fluoroquinolone antibacterial levofloxacin. *J. Med. Chem.* **42**, 4928–4931 https://doi.org/10.1021/jm9904598

266 Campbell, J., Singh, A.K., Santa Maria, J.P., Kim, Y., Brown, S., Swoboda, J.G. et al. (2011) Synthetic lethal compound combinations reveal a fundamental connection between wall teichoic acid and peptidoglycan biosyntheses in *Staphylococcus aureus*. *ACS Chem. Biol.* **6**, 106–116 https://doi.org/10.1021/cb100269f

267 Ejim, L., Farha, M.A., Falconer, S.B., Wildenhain, J., Coombes, B.K., Tyers, M. et al. (2011) Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy. *Nat. Chem. Biol.* **7**, 348–350 https://doi.org/10.1038/nchembio.559

268 Vaara, M. and Vaara, T. (1983) Sensitization of Gram-negative bacteria to antibiotics and complement by a nontoxic oligopeptide. *Nature* **303**, 526–528 https://doi.org/10.1038/303526a0

269 Taylor, P.L., Rossi, L., De Pascale, G. and Wright, G.D. (2012) A forward chemical screen identifies antibiotic adjuvants in *Escherichia coli*. *ACS Chem. Biol.* **7**, 1547–1555 https://doi.org/10.1021/cb300269g

270 Vall-Spinosa, A., Lester, W., Moulding, T., Davidson, P.T. and McClatchy, J.K. (1970) Rifampin in the treatment of drug-resistant *Mycobacterium tuberculosis* infections. *N. Engl. J. Med.* **283**, 616–621 https://doi.org/10.1056/NEJM197009172831202

271 Band, V.I., Hufnagel, D.A., Jaggavarapu, S., Sherman, E.X., Wozniak, J.E., Satola, S.W. et al. (2019) Antibiotic combinations that exploit heteroresistance to multiple drugs effectively control infection. *Nat. Microbiol.* **4**, 1627–1635 https://doi.org/10.1038/s41564-019-0480-z

272 Selkon, J.B., Devadatta, S., Kulkarni, K.G., Mitchison, D.A., Narayana, A.S., Nair, C.N. et al. (1964) The emergence of isoniazid-resistant cultures in patients with pulmonary tuberculosis during treatment with isoniazid alone or isoniazid plus PAS. *Bull. World Health Organ.* **31**, 273–294 PMID: 14253244 https://iris.who.int/handle/10665/267134

273 Bushby, S.R. and Hitchings, G.H. (1968) Trimethoprim, a sulphonamide potentiator. *Br. J. Pharmacol. Chemother.* **33**, 72–90 https://doi.org/10.1111/j.1476-5381.1968.tb00475.x

274 Brochado, A.R., Telzerow, A., Bobonis, J., Banzhaf, M., Mateus, A., Selkrig, J. et al. (2018) Species-specific activity of antibacterial drug combinations. *Nature* **559**, 259–263 https://doi.org/10.1038/s41586-018-0278-9

275 Cacace, E., Kim, V., Varik, V., Knopp, M., Tietgen, M., Brauer-Nikonow, A. et al. (2023) Systematic analysis of drug combinations against Gram-positive bacteria. *Nat. Microbiol.* **8**, 2196–2212 https://doi.org/10.1038/s41564-023-01486-9

276 Du, W., Brown, J.R., Sylvester, D.R., Huang, J., Chalker, A.F., So, C.Y. et al. (2000) Two active forms of UDP-N-acetylglucosamine enolpyruvyl transferase in Gram-positive bacteria. *J. Bacteriol.* **182**, 4146–4152 https://doi.org/10.1128/JB.182.15.4146-4152.2000

277 Blake, K.L., O';Neill, A.J., Mengin-Lecreulx, D., Henderson, P.J.F., Bostock, J.M., Dunsmore, C.J. et al. (2009) The nature of *Staphylococcus aureus* MurA and MurZ and approaches for detection of peptidoglycan biosynthesis inhibitors. *Mol. Microbiol.* **72**, 335–343 https://doi.org/10.1111/j.1365-2958.2009.06648.x

278 Dunsmore, C.J., Miller, K., Blake, K.L., Patching, S.G., Henderson, P.J.F., Garnett, J.A. et al. (2008) 2-Aminotetralones: novel inhibitors of MurA and MurZ. *Bioorg. Med. Chem. Lett.* **18**, 1730–1734 https://doi.org/10.1016/j.bmcl.2008.01.089

279 Kobayashi, K., Ehrlich, S.D., Albertini, A., Amati, G., Andersen, K.K., Arnaud, M. et al. (2003) Essential *Bacillus subtilis* genes. *Proc. Natl Acad. Sci. U.S.A.* **100**, 4678–4683 https://doi.org/10.1073/pnas.0730515100

280 Kock, H., Gerth, U. and Hecker, M. (2004) MurAA, catalysing the first committed step in peptidoglycan biosynthesis, is a target of Clp-dependent proteolysis in *Bacillus subtilis*. *Mol. Microbiol.* **51**, 1087–1102 https://doi.org/10.1046/j.1365-2958.2003.03875.x

281 Kedar, G.C., Brown-Driver, V., Reyes, D.R., Hilgers, M.T., Stidham, M.A., Shaw, K.J. et al. (2008) Comparison of the essential cellular functions of the two murA genes of *Bacillus anthracis*. *Antimicrob. Agents Chemother.* **52**, 2009–2013 https://doi.org/10.1128/AAC.01594-07

282 Edwards, S.L., Brough, R., Lord, C.J., Natrajan, R., Vatcheva, R., Levine, D.A. et al. (2008) Resistance to therapy caused by intragenic deletion in BRCA2. *Nature* **451**, 1111–1115 https://doi.org/10.1038/nature06548

283 Sakai, W., Swisher, E.M., Karlan, B.Y., Agarwal, M.K., Higgins, J., Friedman, C. et al. (2008) Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. *Nature* **451**, 1116–1120 https://doi.org/10.1038/nature06633

284 Szybalski, W. and Bryson, V. (1952) Genetic studies on microbial cross resistance to toxic agents. I. Cross resistance of *Escherichia coli* to fifteen antibiotics. *J. Bacteriol.* **64**, 489–499 https://doi.org/10.1128/jb.64.4.489-499.1952

285 Lázár, V., Pal Singh, G., Spohn, R., Nagy, I., Horváth, B., Hrtyan, M. et al. (2013) Bacterial evolution of antibiotic hypersensitivity. *Mol. Syst. Biol.* **9**, 700 https://doi.org/10.1038/msb.2013.57

286 Minato, Y., Dawadi, S., Kordus, S.L., Sivanandam, A., Aldrich, C.C. and Baughn, A.D. (2018) Mutual potentiation drives synergy between trimethoprim and sulfamethoxazole. *Nat. Commun.* **9**, 1003 https://doi.org/10.1038/s41467-018-03447-x

287 Zheng, J., Rubin, E.J., Bifani, P., Mathys, V., Lim, V., Au, M. et al. (2013) para-Aminosalicylic acid is a prodrug targeting dihydrofolate reductase in *Mycobacterium tuberculosis*. *J. Biol. Chem.* **288**, 23447–23456 https://doi.org/10.1074/jbc.M113.475798

288 Soleimany, A.P., Amini, A., Goldman, S., Rus, D., Bhatia, S.N. and Coley, C.W. (2021) Evidential deep learning for guided molecular property prediction and discovery. *ACS Cent. Sci.* **7**, 1356–1367 https://doi.org/10.1021/acscentsci.1c00546

289 Walsh, C.T. (2019) Chemical biology: here to stay? *Isr. J. Chem.* **59**, 7–17 https://doi.org/10.1002/ijch.201800004