Supplementary Discussion

Several general principles emerge from our data. First, drug-drug interactions are highly species-specific, even if the individual drugs have the same cellular targets across species. This is likely because the underlying mechanisms behind drug-drug interactions are not conserved. Such mechanisms depend on the intracellular wiring between the targeted processes [1,2], but even more on modulating the uptake/efflux of the combined drugs [3], as we demonstrated for several cases. Inter-process wiring is lowly conserved even among closely-related microbial species [4], and both uptake and drug efflux depend on the most diverse part of bacterial cells: their envelope, harboring redundant transport systems, and assembly machineries/enzymes. The consequences of species-specificity for drug-drug interactions are manifold. For antibacterials, this means that narrow-spectrum therapies, constituting a major effort of current and future drug development [5,6], can derive from synergistic combinations of already approved drugs. On the other hand, species-specific antagonisms can potentially be used to mitigate the collateral damage of antibiotic therapies to the gut microbiota [7]. As non-antibiotic drugs also take a high toll on our resident gastrointestinal flora [8,9], such antagonisms may be a more general antidote-strategy for minimizing the adverse impact of drugs on human gut microbiota.

Second, antagonisms and synergies have clearly separable properties. While antagonisms strictly occur between drugs targeting different processes, synergies are more likely for drugs targeting the same processes. This distinction has clear mechanistic base at the drug target level. Disrupting chemically or genetically a process at different steps is known to result in synergistic effects across organisms [2,10,11]. Some of the most robust antibacterial monotherapies come from multi-target drugs inhibiting the same or directly linked processes [12-15]. On the other hand, combining drugs that target distinct core processes may help the organism reaching a more stable equilibrium, as in the case of DNA and protein synthesis inhibitors [1]. Consistently, genetic interactions are more commonly alleviating when genes are part of distinct functional processes in yeast [10].

Third, antagonisms are more prevalent than synergies, demonstrating that if random or empirical mixing of drugs has an effect, this will most likely be a reduction of individual drug efficacies. Even commonly used drug combinations in the clinic, such as linezolid with meropenem in sepsis patients, can have strongly antagonistic effects for some pathogens [16]. Although antagonistic interactions pose efficacy and potentially toxicity issues in the clinic, their use can counter-select resistant isolates [17,18]. On the other hand, synergies are more

conserved than antagonisms across pathogenic species, which is encouraging for clinical use of combinations.

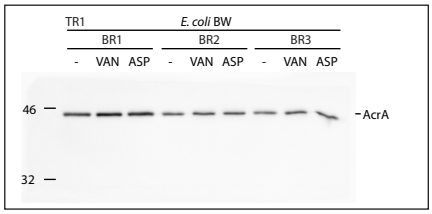
Finally, although antibacterials of the same class had similar interactions with other drugs, most antagonisms we tested were at least partially due to modulation of intracellular drug concentrations. This suggests that drug-drug interactions are only partially driven by MoA and should not be automatically translated as direct functional interactions of their primary targets. This is likely the reason for the low conservation of drug-drug interactions across bacterial species, although their primary targets are highly conserved. Moreover, many antibiotic classes exhibited further subdivisions or members with outlier behaviors. This exposes the risk of drawing general conclusions for an entire class by studying one of its members. Similarly, we did not observe exclusive synergy or antagonism between bactericidal drugs and oxidative stress, suggesting that the interrelation of these different classes of antibiotics and reactive oxygen species may be more complex than previously thought [19,20]. The interactions we report here are at the growth inhibition level. Although we did not probe systematically, 16/16 drug-drug interactions were also detectable at a killing level. More systematic profiling will be required in the future to assess how drug-drug interaction outcomes relate at different levels (inhibition, killing, persister formation).

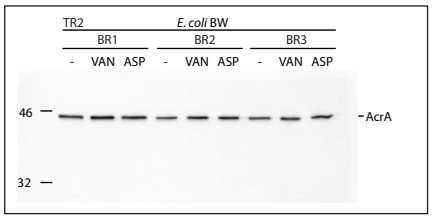
Beyond unraveling general principles, our work provides an unparalleled number of drug-drug interactions in Gram-negative species. We demonstrated the potency of several synergistic pairs against MDR clinical isolates *in vitro*, and for two of them *in vivo*, employing an established insect infection-model. Many more drug pairs are still to be uncovered within our dataset. Interestingly, human-targeted drugs were among the most frequent antibiotic adjuvants in our screen, and although we included only four food additives, we identified 64 synergies, one of which inhibited the growth of MDR *E. coli* isolates. In this particular case vanillin synergized with spectinomycin, because it increased its intracellular concentration, via MdfA, a specific enterobacterial transporter. This narrow-spectrum strong interaction opens the door for reusing an almost neglected antibiotic. Low amounts of vanillin (65 µg/ml) were enough to sensitize the largely resistant *E. coli* to spectinomycin, bringing the MIC from >30 to 10-15 µg/ml, which is similar to MICs of spectinomycin in *Neisseria gonorrhoeae*, against which spectinomycin is still clinically used [21]. Thus, profiling more human-targeted drugs and food additives in future combinatorial screening will not only increase the possible solution space, but may also lead to efficient treatments against MDR pathogens. Since many more human-targeted drugs inhibit bacterial growth than previously appreciated [8], such adjuvant strategies are particularly relevant.

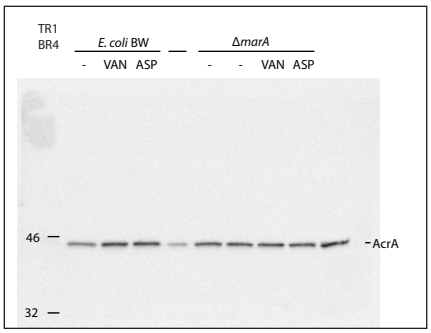
1 Bollenbach, T., Quan, S., Chait, R. & Kishony, R. Nonoptimal microbial response to antibiotics underlies suppressive drug interactions. *Cell* **139**, 707-718, doi:S0092-8674(09)01315-4 [pii] 10.1016/j.cell.2009.10.025 (2009).
2 Nichols, R. J. *et al.* Phenotypic Landscape of a Bacterial Cell *Cell* **144**, 143-156 (2011).
3 Chevereau, G. & Bollenbach, T. Systematic discovery of drug interaction mechanisms. *Mol Syst Biol* **11**, 807, doi:10.15252/msb.20156098 (2015).
4 Ryan, C. J. *et al.* Hierarchical modularity and the evolution of genetic interactomes across species. *Mol Cell* **46**, 691-704, doi:10.1016/j.molcel.2012.05.028 (2012).
5 Butler, M. S., Blaskovich, M. A. & Cooper, M. A. Antibiotics in the clinical pipeline in 2013. *J Antibiot (Tokyo)* **66**, 571-591, doi:10.1038/ja.2013.86 (2013).
6 Brown, E. D. & Wright, G. D. Antibacterial drug discovery in the resistance era. *Nature* **529**, 336-343, doi:10.1038/nature17042 (2016).
7 Blaser, M. J. Antibiotic use and its consequences for the normal microbiome. *Science* **352**, 544-545, doi:10.1126/science.aad9358 (2016).
8 Maier, L. *et al.* Extensive impact of non-antibiotic drugs on human gut commensals *Nature* **555**, 623-628, doi:10.1038/nature25979 (2018).
9 Forslund, K. *et al.* Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. *Nature* **528**, 262-266, doi:10.1038/nature15766 (2015).
10 Costanzo, M. *et al.* A global genetic interaction network maps a wiring diagram of cellular function. *Science* **353**, doi:10.1126/science.aaf1420 (2016).
11 Han, K. *et al.* Synergistic drug combinations for cancer identified in a CRISPR screen for pairwise genetic interactions. *Nat Biotechnol*, doi:10.1038/nbt.3834 (2017).
12 Feng, X. *et al.* Antiinfectives targeting enzymes and the proton motive force. *Proc Natl Acad Sci U S A* **112**, E7073-7082, doi:10.1073/pnas.1521988112 (2015).
13 Ling, L. L. *et al.* A new antibiotic kills pathogens without detectable resistance. *Nature* **517**, 455-459, doi:10.1038/nature14098 (2015).
14 Muller, A. *et al.* Daptomycin inhibits cell envelope synthesis by interfering with fluid membrane microdomains. *Proc Natl Acad Sci U S A*, doi:10.1073/pnas.1611173113 (2016).
15 Daugelavicius, R., Bakiene, E. & Bamford, D. H. Stages of polymyxin B interaction with the *Escherichia coli* cell envelope. *Antimicrob Agents Chemother* **44**, 2969-2978 (2000).
16 Wicha, S. G., Kees, M. G., Kuss, J. & Kloft, C. Pharmacodynamic and response surface analysis of linezolid or vancomycin combined with meropenem against Staphylococcus aureus. *Pharm Res* **32**, 2410-2418, doi:10.1007/s11095-015-1632-3 (2015).
17 Baym, M., Stone, L. K. & Kishony, R. Multidrug evolutionary strategies to reverse antibiotic resistance. *Science* **351**, aad3292, doi:10.1126/science.aad3292 (2016).
18 Yeh, P. J., Hegreness, M. J., Aiden, A. P. & Kishony, R. Drug interactions and the evolution of antibiotic resistance. *Nat Rev Microbiol* **7**, 460-466, doi:nrmicro2133 [pii] 10.1038/nrmicro2133 (2009).
19 Brynildsen, M. P., Winkler, J. A., Spina, C. S., MacDonald, I. C. & Collins, J. J. Potentiating antibacterial activity by predictably enhancing endogenous microbial ROS production. *Nat Biotechnol* **31**, 160-165, doi:10.1038/nbt.2458 (2013).
20 Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A. & Collins, J. J. A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* **130**, 797-810 (2007).
21 Foerster, S., Desilvestro, V., Hathaway, L. J., Althaus, C. L. & Unemo, M. A new rapid resazurin-based microdilution assay for antimicrobial susceptibility testing of *Neisseria gonorrhoeae*. *J Antimicrob Chemother* **72**, 1961-1968, doi:10.1093/jac/dkx113 (2017).

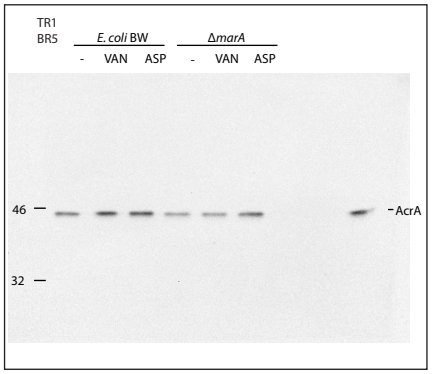
Supplementary Figure - Uncropped scans with size marker indications, related to Figure 3b.

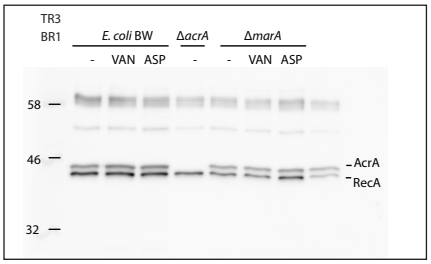
Chemiluminescence images from BioRad ChemiDocTM - Touch gel imaging system for densitometry analysis.

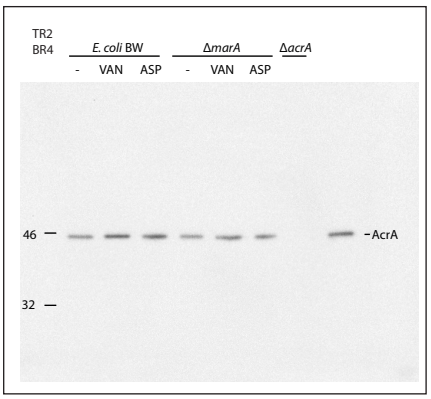


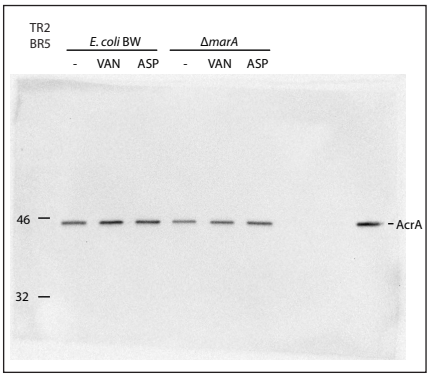












BR = Biological Replicate
TR = Technical Replicate