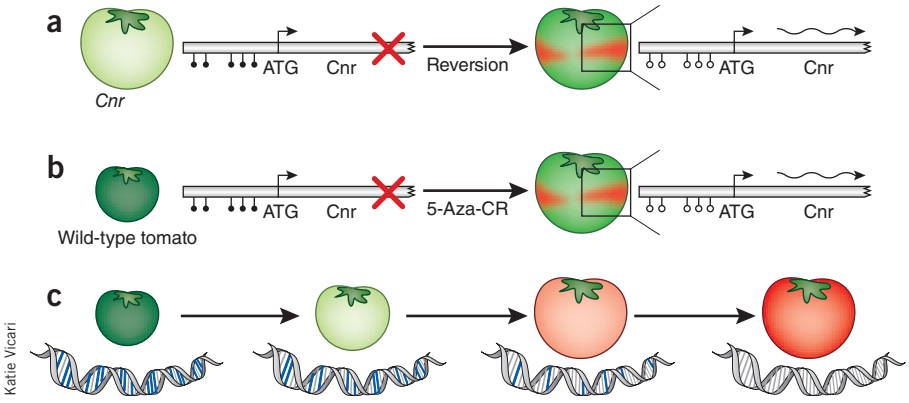

Figure 1 Evidence for epigenetic control of ripening. (**a**) A natural epiallele in *Cnr* prevents ripening, resulting in colorless fruit. The *Cnr* mutant is caused by an epimutation that blocks fruit ripening. Bisulfite sequencing revealed hypermethylation (filled circles) of the *Cnr* promoter, which resulted in inhibition of RIN transcription factor binding, preventing *Cnr* gene expression and fruit ripening. Very rare reversion events result in partial ripening and wild-type sectors (red) in the green fruit. Bisulfite sequencing of the *Cnr* promoter revealed a demethylated state (open circles), allowing binding of RIN to the promoter, *Cnr* expression (wavy arrow) and activation of ripening[3]. (**b**). Unripe tomato fruit is injected with 5-azacytidine, an inhibitor of DNA methylation. Before injection, the *Cnr* gene promoter is hypermethylated, the RIN protein is inhibited from promoter binding and the gene is not transcribed. Thirteen days after drug injection, a time still too early for normal ripening, the previously green tomato is partially ripe (red stripes), indicating drug-induced premature ripening. In ripe tissue (red), *Cnr* is transcribed and the promoter is unmethylated, whereas in adjacent, unripe tissue (green), the *Cnr* promoter is heavily methylated and the gene is not expressed[1]. 5-Aza-CR, 5-azacytidine. (**c**) Progressive states of tomato fruit ripening are accompanied by a developmental program of promoter demethylation in which the promoters of hundreds of fruit ripening genes show a gradual decrease in promoter methylation (indicated in blue in the DNA of the figure), which is accompanied by increased binding of RIN (and other transcription factors) to their promoters and a concomitant increase in RNA expression as fruit ripening progresses[1].

The average methylation level of these genes at RIN binding sites is lower (hypomethlyated) than that of neighboring genomic regions, and methylation further decreases during fruit maturation. Moreover, RIN target gene transcription negatively correlates with the methylation status of RIN binding sites (**Fig. 1c**). These findings are consistent with studies of mammalian genes, where hypomethylation of gene-regulatory regions is commonly observed at sites of DNA-protein interaction[8]. Interestingly, the authors observe very little change in DNA methylation state on transposable elements during fruit maturation, in stark contrast to the novel developmental demethylation events recently reported in the endosperm and pollen of *Arabidopsis*, which occur mainly on transposable elements[9,10].

As with other studies of widespread changes in DNA methylation and gene regulation, the results of Zhong *et al.*[1] are largely correlative, and one should be cautious in drawing conclusions about a cause-and-effect relationship[11]. Nevertheless, three key observations support the hypothesis that genome methylation contributes to repression of fruit ripening before seed maturation: first, promoters of ripening genes become demethylated during development but are hypermethylated in ripening-deficient mutants; second, pharmacological studies reveal that 5-azacytidine induces early ripening; and third, RIN does not bind hypermethylated *Cnr* promoters.

Fortunately, direct testing of the role of DNA methylation during fruit development may soon be made possible by new technologies for epigenome editing. For example, the importance of cytosine methylation in the *Cnr* promoter (or any other promoter) could be tested by fusing proteins that write (methyltransferases) or erase (demethylases) cytosine base modifications to custom-designed DNA binding transcription activator-like effector proteins. Regulated expression of such transgenes in plants might provide a means of targeting cytosine methylation or demethylation events to specific *cis*-elements (e.g., RIN binding sites) in order to assess the functions of epigenetic marks in specific developmental contexts such as ripening. 'Epigenetic engineering' might prove especially useful for trait improvement in crops that have little genetic diversity owing to breeding bottlenecks, such as the domesticated soybean. For breeders, the main outcome of this study is the realization that the identification of epigenetic variation in genes that encode economically important plant traits might provide an important new resource for creating improved crop varieties.

Editor's note: Dr. Ecker reviewed a preprint of this paper before acceptance but had no role in revision of the manuscript.

COMPETING FINANCIAL INTERESTS
The author declares no competing financial interests.

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Discovery of antibiotic adjuvants

Maya A Farha & Eric D Brown

Modeling the production of reactive oxygen species in bacteria reveals targets for adjuvants that boost antibiotic activity.

With the global rise of antibiotic-resistant pathogens, new therapeutic strategies are urgently needed. One approach for improving the efficacy of existing antibiotics and for suppressing the emergence of resistant strains involves the use of antibiotic adjuvants—compounds that make bacteria more susceptible to antibiotics[1]. In this issue, Brynildsen *et al.*[2] identify targets for adjuvants by building on previous findings that bactericidal antibiotics work in part by inducing reactive oxygen species (ROS)[3]. By incorporating ROS production into a genome-scale metabolic model of *Escherichia coli*, the authors predict which genes can be targeted to amplify endogenous

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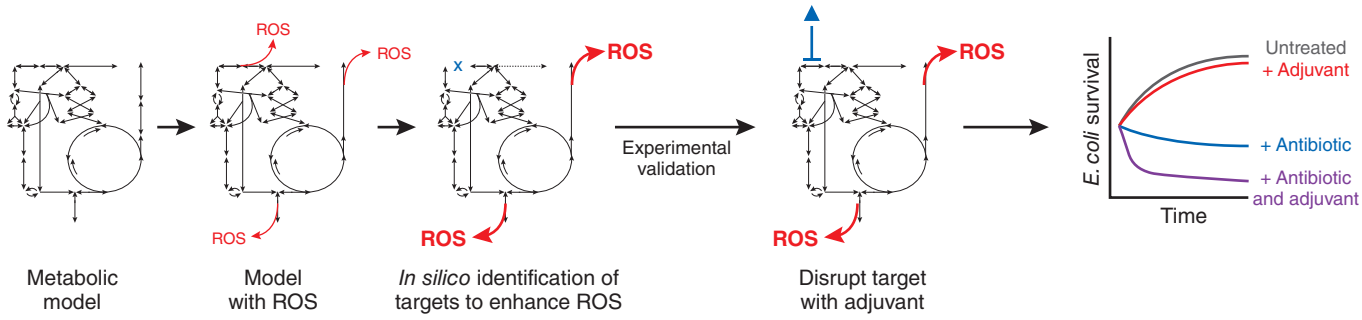

Figure 1 Systems-level approach for identifying novel targets for adjuvants that potentiate the activity of bactericidal antibiotics. Brynildsen *et al.*[2] incorporate reactions that generate ROS into an existing model of *E. coli* metabolism. *In silico* analysis of the effects of gene deletions on ROS production identifies putative ROS modulators that are validated experimentally. Gene deletions that enhance endogenous ROS levels indicate novel adjuvant targets. Small-molecule adjuvants targeting these gene products can enhance the susceptibility of *E. coli* to killing by bactericidal antibiotics.

ROS production and promote bacterial death. Targets identified by this method may lead to new adjuvants with mechanisms of action that differ from those of existing adjuvants.

Although bacterial infections are usually treated by monotherapies, an emerging recognition of the complexity and redundancy of bacterial systems underscores the advantages of using drug combinations[4]. Indeed, antibiotic resistance has been surmounted, *in vitro* at least, by applying combinations of antibiotics and also by combining antibiotics with nonantibiotic compounds that either increase susceptibility to antibiotics[5,6] or interfere with mechanisms of antibiotic resistance[7,8]. Adjuvants can both improve antibiotic efficacy and eliminate or slow the emergence of antibiotic resistance. The archetypal example is the highly successful drug combination Augmentin, containing the β-lactam antibiotic amoxicillin and the β-lactamase inhibitor clavulanic acid. The latter molecule inactivates β-lactamase enzymes that are the key resistance mechanism against β-lactams.

Recently, there has been growing interest in uncovering novel antibiotic adjuvants through systematic approaches. The systems-level approach adopted by Brynildsen *et al.*[2] enables the rapid identification of targets for antibiotic adjuvants that compromise the ability of a bacterial pathogen to survive in the presence of antibiotics that increase oxidative stress. Previous work has shown that bactericidal antibiotics lead to cell death in part by generating ROS[3]. ROS can damage DNA, RNA, proteins and lipids, resulting in cell death when ROS levels exceed the organism's ability to defend itself through its detoxification and repair systems. Brynildsen *et al.*[2] propose to increase antibiotic efficacy not by impairing the organism's ROS defense systems but by amplifying endogenous ROS production, which should compromise its ability to cope with oxidative attack from the antibiotic.

This strategy is one of several for adjuvant therapy that manipulate the physiological state of a microbe to enhance antibiotic susceptibility. In fact, previous work by the same group demonstrated that the use of specific bacterial metabolites can potentiate aminoglycoside antibiotics against bacterial persister cells[9], a small but troublesome fraction of a bacterial population that often survives antibiotic killing. In this earlier work, metabolites were shown to enhance bacterial energetics and, in so doing, to induce uptake of the antibiotics. Others have used adjuvants to enhance the dispersion of biofilms, enriching for planktonic growth, to improve antibiotic sensitivity[10]. The approach of Brynildsen *et al.*[2] is distinctive as it not only takes advantage of a physiological vulnerability common to most microbes but also allows for the discovery of antibiotic adjuvants with unique modes of action.

Genome-scale metabolic models have been under development over the past decade for several clinically important bacterial pathogens, but ROS production has not been quantitatively measured nor have the outcomes of ROS production been assessed on a global level. Brynildsen *et al.*[2] have constructed the first metabolic model capable of estimating ROS production. ROS are generated by many reactions (133 in *E. coli* by the authors' count) functioning in highly interconnected pathways. Thus, predicting targets that will alter ROS production is difficult because perturbing a single enzyme may cause far-reaching changes in the flow of metabolites through the entire metabolic network, altering ROS levels through multiple pathways. The authors overcame this complexity by first specifying new model parameters to represent the rate of ROS production for each possible ROS-generating enzyme. Then, because these parameters vary between enzymes and have not been measured *in vivo*, many potential parameter values were tested in simulations to estimate the most likely effect of a perturbation on ROS levels.

Brynildsen *et al.*[2] began with a previously developed metabolic reconstruction of *E. coli* and added 266 new reactions that could generate the major ROS molecules, 133 for O_2^- and 133 for H_2O_2 (**Fig. 1**). Using flux balance analyses, adapted to meet the requirements of their model, they were able to simulate ROS production and calculate the flow of O_2^- and H_2O_2 in response to genetic perturbations. By systematically analyzing the effects of selected virtual gene deletions on ROS production in their model, they identified the deletions that were most likely to increase ROS production. Most of the predicted ROS modulators were involved in metabolic energy production and represented adjuvant targets with strong potential to increase the activity of bactericidal antibiotics.

To validate their *in silico* analysis, Brynildsen *et al.*[2] measured the levels of O_2^- and H_2O_2 in 21 different *E. coli* strains in which the predicted target genes had been deleted. In most cases, there was good qualitative (up to 90%) agreement between the modeling predictions and the experimental data. The authors next investigated the susceptibility of strains harboring the experimentally validated genetic deletions to the oxidants H_2O_2, O_2^- and NaOCl. Gene-deletion strains with increased basal production of O_2^- or H_2O_2 were generally more susceptible to exogenous oxidative attack, supporting the hypothesis that the predicted genes are adjuvant targets.

The authors also showed that increased microbial ROS production potentiates killing by the bactericidal antibiotics β-lactam and fluoroquinolone. Three of the validated mutant strains, *ΔcyoA*, *ΔnuoG* and *ΔsdhC*, were more sensitive to both antibiotics. Interestingly, enhanced susceptibility to aminoglycoside antibiotics was not detected in the mutant strains. Uptake of aminoglycosides is driven by the proton motive force, which is abolished when ROS concentrations are increased over wild-type levels. Overall, the authors correctly predicted the sensitivity of the mutant strains to both β-lactam and fluoroquinolone antibiotics over 70% of the time.

Notably, the approach succeeded in identifying a new compound with adjuvant activity. Carboxin inhibits the function of succinate dehydrogenase, one of the validated targets identified in the study. The authors found that carboxin, which has not been used as an adjuvant before, was synergistic with ampicillin, increasing both production of H_2O_2 and bactericidal activity by approximately tenfold over ampicillin alone.

The study of Brynildsen *et al.*[2] highlights the utility of metabolic modeling for antibiotic drug discovery. One obstacle to this approach, however, is the complexity of bacterial metabolism. The absence of regulatory and kinetic information in such models limits the scope and accuracy of predictions. With more comprehensive knowledge of microbial systems, genome-scale models will surely become increasingly important in identifying microbial targets for both mono- and combination therapies.

Despite the promise of this work, it is worth considering that the identified targets are involved exclusively in respiration, either through the production or use of ATP. This raises the issue of whether the approach will be clinically useful, because agents that target respiratory enzymes are often harmful to human health[11]. Carboxin, the adjuvant studied here, is a fungicide that is used in agriculture but is toxic to humans. Thus, the main challenge in developing antibiotic adjuvants will be to achieve bacterial selectivity.

The authors' ingenious use of metabolic modeling to find synergies with existing antibiotics might also be broadly applicable to pathogens other than *E. coli*. ROS are ubiquitous in bacteria exposed to aerobic environments, and as such, this tactic should be translatable to any pathogen for which a metabolic reconstruction is available, provided it is not a strict anaerobe. Although the study provides compelling evidence that the approach is valid with β-lactam and fluoroquinolone antibiotics, it remains to be seen whether it is applicable to other classes of bactericidal agents, such as the cell-wall inhibitors vancomycin and bacitracin and the polymyxin antibiotics. Bacteriostatic antibiotics do not generate ROS as part of their mechanism of action[3], and, indeed, Brynildsen *et al.*[2] found that their activity is unaffected by increases in ROS levels.

Adjuvant strategies could revitalize antibiotic therapy both by potentiating drug activity and by slowing the emergence of antibiotic resistance. Such approaches would be synergistic with a renewed emphasis on multicomponent therapies to combat the increasing prevalence of antibiotic-resistant bacteria.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Pig genomics for biomedicine

Randall S Prather

The newly released swine genome assembly will aid research on disease models and xenotransplantation.

Pigs provide an important source of protein for a growing world population and are increasingly appreciated for their potential to model human disease and to supply organs for transplantation therapy in humans. With the recent publication of the first high-quality draft swine reference genome in *Nature*[1], efforts to extend the usefulness of pigs in agriculture and biomedicine have acquired an essential resource (**Fig. 1**).

The US National Institutes of Health deemed the pig so important for biomedical research that it established the National Swine Resource and Research Center to serve as a genetic resource and repository for disease models and as a core facility to create new genetic mutants (http://nsrrc.missouri.edu/). Swine have long been recognized as an especially good model for certain human diseases[2]. For example, modification of the *CFTR* gene results in a phenotype resembling human cystic fibrosis in pigs but not in mice[3]. Pigs develop atherosclerosis and lay down plaques in a manner similar to humans. And owing to the similarity of photoreceptor distribution and of the size of pig and human eyes, these animals are an excellent model of human eye disease[2]. The advantages of pigs as disease models likely derive from their genetic similarity to humans;

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