Glyphosate perturbs the gut microbiota of honey bees

Erick V. S. Motta[a,1], Kasie Raymann[a,2], and Nancy A. Moran[a,1]

[a]Department of Integrative Biology, University of Texas at Austin, Austin, TX 78712

Edited by Margaret J. McFall-Ngai, University of Hawaii at Manoa, Honolulu, HI, and approved August 21, 2018 (received for review March 6, 2018)

Glyphosate, the primary herbicide used globally for weed control, targets the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme in the shikimate pathway found in plants and some microorganisms. Thus, glyphosate may affect bacterial symbionts of animals living near agricultural sites, including pollinators such as bees. The honey bee gut microbiota is dominated by eight bacterial species that promote weight gain and reduce pathogen susceptibility. The gene encoding EPSPS is present in almost all sequenced genomes of bee gut bacteria, indicating that they are potentially susceptible to glyphosate. We demonstrated that the relative and absolute abundances of dominant gut microbiota species are decreased in bees exposed to glyphosate at concentrations documented in the environment. Glyphosate exposure of young workers increased mortality of bees subsequently exposed to the opportunistic pathogen *Serratia marcescens*. Members of the bee gut microbiota varied in susceptibility to glyphosate, largely corresponding to whether they possessed an EPSPS of class I (sensitive to glyphosate) or class II (insensitive to glyphosate). This basis for differences in sensitivity was confirmed using in vitro experiments in which the EPSPS gene from bee gut bacteria was cloned into *Escherichia coli*. All strains of the core bee gut species, *Snodgrassella alvi*, encode a sensitive class I EPSPS, and reduction in *S. alvi* levels was a consistent experimental result. However, some *S. alvi* strains appear to possess an alternative mechanism of glyphosate resistance. Thus, exposure of bees to glyphosate can perturb their beneficial gut microbiota, potentially affecting bee health and their effectiveness as pollinators.

honey bees | microbiome | glyphosate | *Snodgrassella alvi* | *Serratia*

The broad-spectrum herbicide glyphosate [*N*-(phosphonomethyl)glycine] has long been the primary weed management system, and its use is growing in connection with crops genetically engineered to be resistant to glyphosate (1, 2). Its mechanism of action, inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme in the shikimate pathway, prevents the biosynthesis of aromatic amino acids and other secondary metabolites in plants and some microorganisms (3). EPSPS catalyzes the reaction between phosphoenolpyruvate (PEP) and shikimate 3-phosphate (S3P) (4), and glyphosate is a competitive inhibitor that blocks the PEP-binding site (5). EPSPS enzymes from different organisms vary in molecular weight (46–178 kDa) and sequence homology (6) and form two phylogenetic clusters that differ in tolerance to glyphosate. Class I enzymes are sensitive to glyphosate and are present in all plants and in some bacteria, such as *Escherichia coli* (4); class II enzymes are only found in some bacteria, such as *Staphylococcus aureus*, and can tolerate high concentrations of glyphosate (7, 8).

Animals lack the shikimate pathway, which is why glyphosate is considered one of the least toxic pesticides used in agriculture (9). However, some evidence suggests that glyphosate affects nontarget organisms, for example, changing the behavior of honey bees (10), reducing reproduction of soil-dwelling earthworms (11), and affecting the growth of microalgae and aquatic bacteria (12). Glyphosate is also associated with changes in plant endophytic and rhizosphere microbiomes (2) and with disturbances of gut microbiota of animals living near agricultural sites (13).

Honey bees and bumble bees are major pollinators of flowering plants, including many crops. When foraging, they can be exposed to a variety of xenobiotics, such as glyphosate. This herbicide is known to affect the growth of microorganisms (13–15), and the health of bees is intrinsically related to their distinct gut microbial community (16, 17). The honey bee gut microbiota is dominated by eight bacterial species: *Lactobacillus* spp. Firm-4, *Lactobacillus* spp. Firm-5 (phylum Firmicutes), *Bifidobacterium* spp. (phylum Actinobacteria), *Snodgrassella alvi*, *Gilliamella apicola*, *Frischella perrara*, *Bartonella apis*, and Alpha 2.1 (phylum Proteobacteria) (18). Each of these species exhibits strain diversity corresponding to differences in metabolic capabilities and tolerances to xenobiotics (19, 20). Newly emerged workers (NEWs) are nearly free of gut bacteria and acquire their normal microbial community orally through social interactions with other workers during the first few days after emergence (21). Bees deprived of their normal microbiota show reduced weight gain and altered metabolism (22), increased pathogen susceptibility (17), and increased mortality within hives (23).

In this study, we investigated the effects of glyphosate exposure on the size and composition of the honey bee gut microbiome. We found the microbiome was affected by glyphosate exposure during and after gut colonization, and that glyphosate exposure during early gut colonization increased mortality of bees exposed to an opportunistic pathogen. Additionally, bee gut bacteria differ in glyphosate susceptibility. We explored the molecular mechanisms of this variability in glyphosate tolerance by expressing the EPSPS of bee gut symbionts in *E. coli*. Some bee gut bacteria tolerate glyphosate by virtue of a class II EPSPS, but a few strains with susceptible class I EPSPS depend on other,

Significance

Increased mortality of honey bee colonies has been attributed to several factors but is not fully understood. The herbicide glyphosate is expected to be innocuous to animals, including bees, because it targets an enzyme only found in plants and microorganisms. However, bees rely on a specialized gut microbiota that benefits growth and provides defense against pathogens. Most bee gut bacteria contain the enzyme targeted by glyphosate, but vary in whether they possess susceptible versions and, correspondingly, in tolerance to glyphosate. Exposing bees to glyphosate alters the bee gut community and increases susceptibility to infection by opportunistic pathogens. Understanding how glyphosate impacts bee gut symbionts and bee health will help elucidate a possible role of this chemical in colony decline.

Author contributions: E.V.S.M. and N.A.M. designed research; E.V.S.M. and K.R. performed research; E.V.S.M., K.R., and N.A.M. analyzed data; and E.V.S.M., K.R., and N.A.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

Data deposition: All new sequence data are available on NCBI BioProject (accession nos. PRJNA432210 and PRJNA480015).

[1]To whom correspondence may be addressed. Email: erickvsm@utexas.edu or nancy.moran@austin.utexas.edu.

[2]Present address: Department of Biology, University of North Carolina at Greensboro, Greensboro, NC 27403.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1803880115/-/DCSupplemental.

yet unknown, mechanisms for tolerance. Overall, our results show that glyphosate exposure can perturb the gut microbiota of honey bees, and that compositional shifts typically favor species tolerant to glyphosate and disfavor sensitive species.

Results and Discussion

Glyphosate Perturbs the Honey Bee Gut Bacterial Community. Hundreds of adult worker bees were collected from a single hive, treated with either 5 mg/L glyphosate (G-5), 10 mg/L glyphosate (G-10) or sterile sucrose syrup (control) for 5 d, and returned to their original hive. Bees were marked on the thorax with paint to make them distinguishable in the hive. Glyphosate concentrations were chosen to mimic environmental levels, which typically range between 1.4 and 7.6 mg/L (24), and may be encountered by bees foraging at flowering weeds. To determine the effects of glyphosate on the size and composition of the gut microbiome, 15 bees were sampled from each group before reintroduction to the hive (day 0) and postreintroduction (day 3), and relative and absolute abundances of gut bacteria were assessed using deep amplicon sequencing of the V4 region of the bacterial 16S rRNA gene and quantitative PCR (qPCR).

At day 0, glyphosate exposure had little effect on the bee gut microbiome size, but the absolute and relative abundances of the core species, *S. alvi*, were significantly lower in the G-10 group (Fig. 1 and *SI Appendix*, Fig. S1). The effects of glyphosate exposure on the bee gut microbiome were more prominent at day 3, after treated bees were returned to the hive. The total number of gut bacteria decreased for both treatment groups, relative to control, but this drop was significant only for the G-5 group, which also exhibited more severe compositional shifts (Fig. 1). The absolute abundances of four dominant gut bacteria, *S. alvi*, *Bifidobacterium*, *Lactobacillus* Firm-4 and Firm-5 were decreased (Fig. 1), and the relative abundance of *G. apicola* increased in the G-5 group (*SI Appendix*, Fig. S1). Surprisingly, only *Lactobacillus* Firm-5 decreased in absolute abundance in the G-10 group (Fig. 1). This experiment was repeated using bees from a different hive and season, and similar trends were observed (*SI Appendix*, Fig. S2). As in the first experiment, significant reductions in abundance were observed for *S. alvi* in bees treated with glyphosate (*SI Appendix*, Fig. S2).

The relative lack of effects of the G-10 treatment on the microbiota composition at day 3 posttreatment is unexplained, but may reflect other effects of glyphosate on bees. Our recapture method fails to sample bees that died or abandoned the hive. Since bees exposed to glyphosate may exhibit impaired spatial processing, compromising their return to hives (10, 24), bees in the G-10 group that consumed more glyphosate-laced sugar syrup before reintroduction to the hive may have been less likely to return to the hive after foraging. Since fewer than 20% of bees

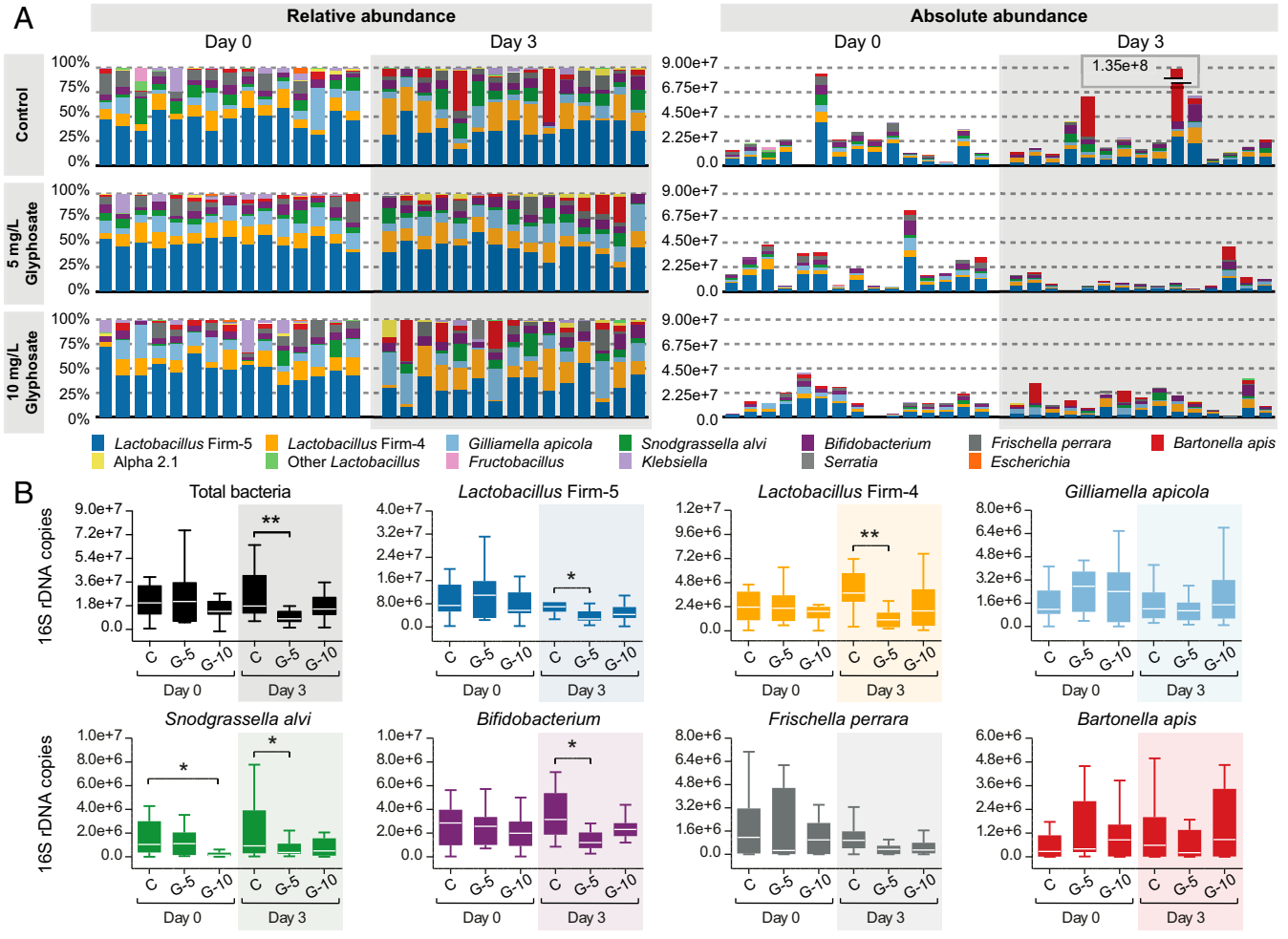


Fig. 1. Changes in gut microbiota composition following glyphosate exposure of honey bees with established gut communities. (*A*) Stacked column graph showing the relative and absolute abundances of gut bacterial species in control bees and bees treated with 5 mg/L or 10 mg/L glyphosate at posttreatment days 0 and 3. Each column represents one bee. (*B*) Boxplots of bacterial 16S rDNA copies for control (C) and glyphosate-treated (G-5 and G-10) bees at posttreatment days 0 and 3 ($n = 15$ for each group and time point). Box-and-whisker plots show high, low, and median values, with lower and upper edges of each box denoting first and third quartiles, respectively. $^*P < 0.05$ and $^{**}P < 0.01$, Wilcoxon rank sum test followed by Bonferroni correction.

reintroduced to the hive were recovered, recovered bees may not represent the total effect of glyphosate on treatment groups.

Glyphosate Affects Early Gut Bacterial Colonization. Glyphosate arrests bacterial growth without directly killing the cells, so we hypothesized that it would have a greater effect on actively dividing cells present during early gut colonization. To test this, NEWs, which are nearly free of gut bacteria (21), were simultaneously exposed to an inoculum consisting of their normal microbial community and to glyphosate. This simultaneous exposure is relevant to field situations, since glyphosate has been detected in hives and honey samples (25, 26), indicating that honey bee foragers can transport residues of this herbicide to the colony and contaminate other bees, including NEWs, and food resources. Also, glyphosate is a stable, water-soluble chemical that can persist in the environment for long periods (10).

Assessment of gut microbiomes, as described in the previous section, identified all eight core gut taxa in both control and treatment groups (Fig. 2*A*), showing that glyphosate does not eliminate colonization by any core member. Average total bacterial abundance was slightly lower in glyphosate-treated bees, but this was not statistically significant (Fig. 2*B*). *S. alvi* was the most strongly affected member of the gut microbiota and decreased in both absolute and relative abundance, while *Lactobacillus* Firm-4 increased in relative abundance (Fig. 2 *C–E* and *SI Appendix*, Fig. S3). Based on relative abundance, gut community compositions of glyphosate-treated bees differed from those of controls (principal coordinate analysis of weighted UniFrac) (27), permanova test with 9,999 permuations; $P = 0.0078$, pseudo-F statistic = 6.66) (Fig. 2*F*). Thus, glyphosate exposure during early development of the gut community can interfere with normal colonization by altering the abundance of beneficial bacterial species.

Typically, captive honey bees do not defecate, and dead bacterial cells and the released DNA accumulate in the gut (23). Thus, we also analyzed changes in bacterial abundance after glyphosate exposure by extracting both DNA and RNA from the guts of treatment and control bees in a second colonization experiment. We included a positive control group, in which bees were exposed to tylosin, an antibiotic used in beekeeping. This antibiotic treatment was expected to perturb the microbiota, but the decrease was significant only for RNA samples (*SI Appendix*, Fig. S4). Glyphosate exposure resulted in nonsignificant decreases in total bacteria for both DNA and RNA assays. We also checked changes in absolute abundance for three core bacterial species, *S. alvi*, *Lactobacillus* Firm-4, and *Lactobacillus* Firm-5. Tylosin treatment resulted in reductions for 16S rRNA copies (*SI Appendix*, Fig. S4). Effects of glyphosate treatment were specific to *S. alvi*, which was the only assayed species showing significant reductions in absolute abundance, observed for both DNA and RNA assays (*SI Appendix*, Fig. S4). This experiment suggests that measures based on DNA are partly obscured by DNA from dead bacterial cells, although this effect does not entirely mask shifts in bacterial abundance.

Glyphosate Exposure Makes Young Worker Bees More Susceptible to *Serratia*. To determine whether glyphosate-induced perturbation of microbiota colonization affects host health, we measured the susceptibility of glyphosate-treated bees to an opportunistic bacterial pathogen. NEWs were exposed to glyphosate in the stage of acquiring their normal microbial community. After 5 d of treatment, bees were challenged with *Serratia marcescens* kz19, an opportunistic pathogen commonly detected at very low frequencies in the bee gut (28, 29).

For bees lacking gut microbiota, *Serratia* challenge resulted in increased mortality relative to that observed for bees with a conventional gut microbiota, regardless of glyphosate exposure (Fig. 2*G* and *SI Appendix*, Fig. S5). For bees with a conventional gut microbiota, glyphosate treatment resulted in increased mortality after *Serratia* challenge. To determine whether this increased mortality was attributable to the effects of glyphosate on the gut microbiota or to direct effects of glyphosate on bees, we included

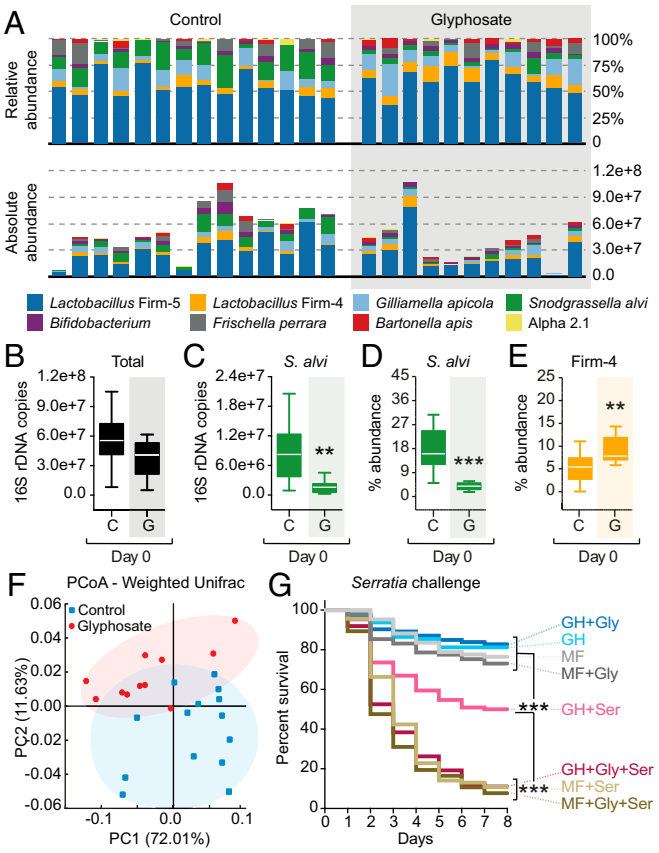


Fig. 2. Changes in gut microbiota composition following glyphosate exposure of young honey bees and susceptibility to *Serratia* infection. (*A*) Stacked column graph showing the relative and absolute abundances of gut bacterial species in control and glyphosate-treated bees. Each column represents one bee. (*B–E*) Boxplots of total bacterial 16S rDNA copies and of absolute and relative abundances of two gut bacterial species for control ($n = 14$) and glyphosate-treated ($n = 11$) bees. $^{**}P < 0.01$, and $^{***}P < 0.001$, Wilcoxon rank sum test followed by Bonferroni correction. (*F*) Principal coordinate analysis of gut community composition using weighted UniFrac (permanova test with 9,999 permuations; $P = 0.0078$, pseudo-F statistic = 6.66). (*G*) The percent survival of age-controlled bees after *Serratia* kz19 exposure, shown as a Kaplan–Meier survival curve. $^{***}P < 0.001$, coxph model implemented in the "survival" package in R. GH, gut homogenate-exposed bees; Gly, glyphosate treatment; MF, microbiota-free bees; Ser, *Serratia* challenge.

control groups not challenged with *Serratia*. In bees exposed to glyphosate, but not challenged with *Serratia*, survival rates were not significantly affected by glyphosate and were much higher than in the *Serratia*-challenged groups (Fig. 2*G*), demonstrating that a direct effect of glyphosate on bees is not the basis of the high mortality of glyphosate-exposed, pathogen-challenged bees.

Our results show that glyphosate reduces the protective effect of the gut microbiota against opportunistic pathogens and that *S. alvi* is the bacterial species most negatively affected by glyphosate exposure. By itself, *S. alvi* appears to give some protection, but not as much as the whole gut microbiota (*SI Appendix*, Fig. S6). *S. alvi* forms a biofim on the wall of the gut ileum (18, 21, 30), which may function as a mechanical barrier against pathogen invasion. Some *S. alvi* strains encode type VI secretion systems (31), which could contribute to colonization resistance through contact-dependent inhibition of *Serratia*. Furthermore, host expression of antimicrobial peptides is upregulated after *S. alvi* colonization (32), which could increase resistance to infection by pathogens. Besides a direct protective effect, *S. alvi* may be critical in enabling the full microbiota to assemble, thus enabling greater protection.

The Bee Gut Contains Bacterial Species with both Sensitive and Insensitive Types of EPSPS. Bacterial EPSPS exists as two main types, corresponding to two phylogenetic clusters, that differ in sensitivity to glyphosate: Class I is naturally sensitive, whereas class II is insensitive (8). To identify the EPSPS types present in the bee gut microbiota, a phylogenetic tree was constructed using the EPSPS protein of bacterial strains isolated from honey bee and bumble bee guts and of other representative organisms (Fig. 3). EPSPS sequences from *S. alvi*, *G. apicola*, *F. perrara*, *Bifidobacterium*, and *Apibacter adventoris* (phylum Bacteroidetes) (33) clustered with those from other organisms containing a class I EPSPS, and thus these bacteria are predicted to be sensitive to glyphosate. In contrast, sequences from *B. apis* and *Lactobacillus* Firm-4 clustered with other bacteria containing a class II EPSPS, as did *Parasaccharibacter apium* (Alpha 2.2), a bacterium commonly detected in honey bee larvae and hives, but rare in the guts of workers (34), and *Paenibacillus larvae*, the agent of American foulbrood in honey bee larvae (35); these bacteria are predicted to be unaffected by glyphosate exposure. *Lactobacillus* Firm-5 strains for which genomes are available lack the gene encoding EPSPS and were excluded from our analysis.

Bee Gut Bacteria Vary in Glyphosate Sensitivity at the Species and Strain Levels. Several bee gut-associated bacterial strains isolated from honey bees and bumble bees were cultured in vitro in the presence or absence of a high dose of glyphosate. Most *S. alvi* and *G. apicola* strains tested, which contain a class I EPSPS, either do not grow or have a delay in growth when cultured in the presence of glyphosate; no such effect is observed for strains containing a class II EPSPS, *Lactobacillus* Firm-4 and *B. apis* (Fig. 3 and *SI Appendix*, Fig. S7). However, *S. alvi* strains wkB2 and wkB298, despite containing a class I EPSPS, grow as well in the presence of glyphosate as in its absence, with no initial delay in growth. We looked for potential single-site mutations in the EPSPS active site of these strains, which is known to confer tolerance to glyphosate (36), but no mutations were observed, indicating that the resistance in these *S. alvi* strains results from other mechanisms.

A previous study of the genes required by *S. alvi* to live in the bee hindgut showed that the aromatic amino acid biosynthetic pathway is required for growth in this niche (30), which is consistent with low aromatic amino acid concentrations in the hindgut (37). Thus, bee gut bacterial strains having a glyphosate-susceptible EPSPS are predicted to drop in abundance following exposure, as observed for *S. alvi* (Fig. 1 and *SI Appendix*, Fig. S2) and *Bifidobacterium* (Fig. 1) in the hive experiments. *Lactobacillus* Firm-4, which encodes a class II EPSPS, and Firm-5, which does not contain the target enzyme of glyphosate, also had their abundances reduced in the hive experiment (Fig. 1), which was not expected. This may be explained by the fact that these strains lack the aromatic amino acid biosynthetic pathway (18), relying on uptake of aromatic amino acids released by other bacterial species, such as *S. alvi*, in the bee gut environment. The increase in *G. apicola* relative abundance (*SI Appendix*, Fig. S1) was unpredicted, but was also observed in a previous study on microbial community responses to antibiotic perturbation (23).

Glyphosate Resistance Is Independent of EPSPS Class in Some Bee Gut Strains. To understand the mechanism that prevents some bee gut bacterial strains from growing in the presence of glyphosate, we complemented *E. coli ΔaroA* with *aroA* genes cloned from bee gut bacterial strains as well as with the *E. coli* K12 *aroA*, which is known to be sensitive to glyphosate. *E. coli ΔaroA* cannot synthesize aromatic amino acids and does not grow in minimal media, but grows normally when transformed with an arabinose-inducible plasmid carrying the intact *E. coli aroA* gene (Fig. 4).

Transformants carrying the *aroA* gene from *S. alvi*, *G. apicola*, and *B. apis* were able to grow in minimal media at a similar rate to the transformant carrying the *aroA* gene from *E. coli* (Fig. 4). The addition of 10 mM glyphosate to the media resulted in a delay in growth of ~48–72 h for transformants carrying the *aroA* gene from

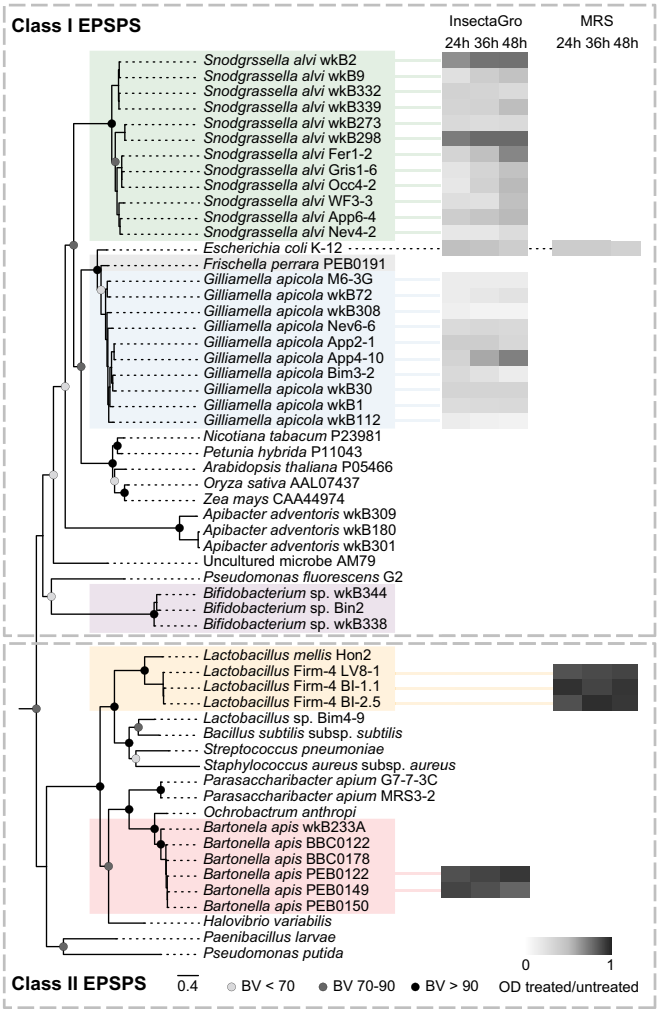


Fig. 3. Maximum-likelihood phylogeny based on amino acid sequences of EPSPS (PhyML 3.1, LG model + Gamma4, 100 bootstrap replicates). Bee gut bacterial strains, other bacteria, and some plant species are represented in the phylogeny. The heatmap represents the growth of some bee bacterial strains in the presence/absence of 10 mM glyphosate at three time points (24, 36, and 48 h). Glyphosate was dissolved in the culture media (InsectaGro or MRS broth, based on bacterial preferences). A value of 1 indicates that growth is the same in the presence or absence of glyphosate.

all *S. alvi* and *G. apicola* strains tested (Fig. 4). This is expected if glyphosate binds to a susceptible EPSPS, blocking the shikimate pathway and preventing bacterial growth until the concentration of PEP or EPSPS exceeds that of glyphosate, allowing the transformants to resume growth. On the other hand, the transformant carrying the *aroA* gene from *B. apis* did not exhibit the growth delay in the presence of glyphosate (Fig. 4), as predicted since this *aroA* version encodes an insensitive class II EPSPS. Moreover, the addition of increased concentrations of arabinose in the media or reduction in glyphosate concentration sped up the growth of all transformant strains (*SI Appendix*, Fig. S8), which corroborates the reversible mechanism of EPSPS inhibition by glyphosate.

Although *S. alvi* strains wkB2 and wkB298 were resistant to glyphosate (Fig. 3 and *SI Appendix*, Fig. S7), their *aroA* versions were sensitive to glyphosate (Fig. 4). Potentially, some bee gut microbes may have evolved alternative glyphosate resistance mechanisms due to a history of exposure, similar to the resistance observed for the antibiotic tetracycline used in beekeeping (38). Therefore, we overexpressed, in WT *E. coli*, certain genes encoding transporters from some bee gut bacteria, including wkB2 and wkB298 strains, that could be involved in

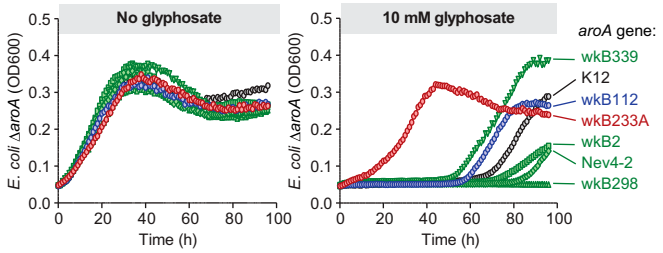


Fig. 4. Growth curves of *E. coli* Δ*aroA* BW25113 expressing the *aroA* gene from different bee-associated bacterial strains (*B. apis* in red, *E. coli* in black, *G. apicola* in blue, and *S. alvi* in green) cultured in minimal media in the presence or absence of 10 mM glyphosate.

glyphosate resistance: *yhhS*, which encodes a membrane transporter conferring glyphosate tolerance when overexpressed in *E. coli* (39), and *tetC*, which encodes an efflux pump that provides tetracycline resistance to *S. alvi* wkB2 (38). However, these transporters were not able to reverse the delay in *E. coli* growth caused by glyphosate (*SI Appendix*, Fig. S9). As the glyphosate tolerance exhibited by some *S. alvi* strains does not appear to be due to a resistant EPSPS or to transport by YhhS or TetC, these strains are likely to employ a novel mechanism of glyphosate resistance. Future studies might identify this mechanism and determine the evolutionary origin of resistance.

***S. alvi* Strains May Vary in Sensitivity to Glyphosate in Vivo.** Our phylogenetic analysis and cloning experiments demonstrated that, despite displaying variable susceptibility to glyphosate in vitro, all *S. alvi* strains possess a glyphosate-sensitive class I EPSPS. Therefore, we investigated whether this variation in susceptibility occurs in vivo. NEWs were monoinoculated with two different *S. alvi* strains: wkB2, which grows in the presence of high concentrations of glyphosate, or wkB339, which exhibits a delay in growth in the presence of high concentrations of glyphosate (Fig. 5*A*). Bees were hand fed bacterial suspensions to inoculate with a control number of *S. alvi* cells, exposed to glyphosate for 3 d, and sampled at days 1 and 3 during exposure. Both *S. alvi* wkB2 and *S. alvi* wkB339 increased in abundance between days 1 and 3 in unexposed bees, consistent with previous findings that *S. alvi* can colonize guts of monoinoculated bees (30, 40). Based on qPCR estimates of *S. alvi* abundance on day 3, glyphosate exposure had a negative effect on growth of both strains [two-way analysis of variance (ANOVA), treatment effect, $P < 0.0001$]. *S. alvi* wkB339 was more affected by glyphosate exposure, based on significant interaction between strain and treatment (two-way ANOVA, $P < 0.0204$). Correspondingly, the absolute abundance of the glyphosate-sensitive strain, wkB339, was significantly lower in glyphosate-treated bees compared with controls (Tukey's test, $P < 0.001$) or wkB2-treated bees (Tukey's test, $P < 0.001$) (Fig. 5 *B* and *C*). Potentially, strain differences in glyphosate sensitivity may contribute to the observed variation in the overall decrease in *S. alvi* abundance when bees with their native gut microbiota are exposed to glyphosate.

Conclusion

As in many animals, honey bees rely on their gut microbial community for a variety of functions, including food processing (25, 26), regulation of immune system (33, 34), and defense against pathogens (17, 27). Perturbations of this system have the potential to lead to negative consequences for host fitness. We found that glyphosate affects the bee gut microbiota composition and that bacterial species and strains within this community vary in susceptibility to glyphosate. Recent experimental and observational studies have provided evidence that dysbiosis affecting the bee gut can increase susceptibility to pathogen invasion (23, 41, 42). Our results also suggest that establishment of a normal microbial community is crucial for protection against opportunistic pathogens of honey bees. Furthermore, our results highlight one potential mechanism by which glyphosate affects bee health.

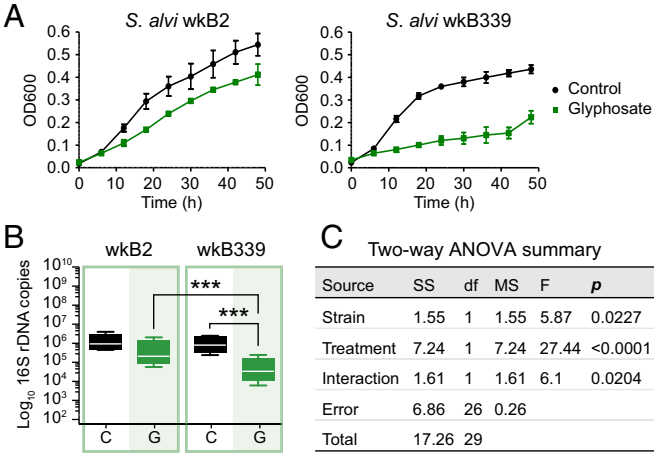
While some species in the bee gut can tolerate high concentrations of glyphosate due to the presence of a class II EPSPS enzyme, others are sensitive due to the presence of a class I EPSPS. A consistent effect of glyphosate on the bee gut microbiota was a negative impact on growth of *S. alvi*, which possesses a sensitive EPSPS. However, some strains of *S. alvi* may tolerate glyphosate through an as yet unknown mechanism. Since bee gut symbionts affect bee development, nutrition, and defense against natural enemies, perturbations of these gut communities may be a factor making bees more susceptible to environmental stressors including poor nutrition and pathogens.

Methods

More details are provided in *SI Appendix*.

Effects of Glyphosate on the Honey Bee Gut Microbiome. Adult workers with established gut communities were collected from a hive at University of Texas, Austin (UT Austin), marked on the thorax with paint, fed glyphosate (5 or 10 mg/L) or sterile sucrose syrup for 5 d, and returned to the same hive. Fifteen bees from each group were sampled before and 3 d after reintroduction to the hive. This experiment was repeated using bees from a different hive and different year. DNA was extracted from dissected guts and used as template for qPCR analyses. DNA samples from the first experiment were submitted for Illumina sequencing at the Genomic Sequencing and Analysis Facility (GSAF) at UT Austin. Illumina sequence reads were processed using QIIME 1.9.1 (43).

Effects of Glyphosate on Early Gut Colonization and Susceptibility to *Serratia* Infection. Hundreds of late-stage pupae were removed from brood frames and allowed to emerge under sterile conditions in laboratory. (Experiment A) NEWs were exposed to bee gut homogenate for 5 d, then hand fed 1 mM glyphosate or sterile sugar syrup on 2 alternate days. Fifteen bees from each group were sampled 2 d after the last hand feeding. DNA was extracted from dissected guts, used as template for qPCR analyses, and submitted for Illumina sequencing at the GSAF, UT Austin. (Experiment B) NEWs were exposed to a bee gut homogenate or sterile sucrose syrup. Each group was divided into two subgroups and treated with 0.1 mM glyphosate or sterile sucrose syrup for 5 d. After that, half of the subgroups was exposed to the opportunistic pathogen *S. marcescens* kz19, whereas the other half was used as controls.



Source	SS	df	MS	F	p
Strain	1.55	1	1.55	5.87	0.0227
Treatment	7.24	1	7.24	27.44	<0.0001
Interaction	1.61	1	1.61	6.1	0.0204
Error	6.86	26	0.26		
Total	17.26	29			

Fig. 5. Variation in *S. alvi* strain sensitivity to glyphosate. (*A*) Growth curves of *S. alvi* wkB2 and wkB339 cultured in InsectaGro media in the presence or absence of 10 mM glyphosate. Experiment was performed in triplicate, and each data point represents the average optical density (600 nm, with SD bars). (*B*) Boxplots of *S. alvi* wkB2 and wkB339 abundances in bees exposed or not to 0.1 mM glyphosate for 3 d estimated by qPCR. Box-and-whisker plots show high, low, and median values, with lower and upper edges of each box denoting first and third quartiles, respectively. ***$P < 0.001$, two-way ANOVA with Tukey's correction for multiple comparisons. (*C*) Two-way ANOVA for effects of *S. alvi* strain and glyphosate treatment.

Bees were exposed to similar amounts of glyphosate (~1.7 μg) in experiments A and B.

***S. alvi* Colonization During Glyphosate Exposure.** NEWs were hand fed 5 μL sucrose syrup containing ~10^5 cells of *S. alvi* wkB2 or wkB339 or sterile sucrose syrup as control. Each group was divided into two subgroups and treated with 0.1 mM glyphosate or sterile sucrose syrup for 3 d immediately following bacterial exposure. Eight bees were sampled from each subgroup at days 1 and 3, and DNA was extracted from dissected guts. *S. alvi*-specific primers (44) were used to amplify total copies of 16S rDNA of each sample by qPCR.

In Vitro Experiments with Bee Gut Bacterial Strains. Honey bee and bumble bee gut bacterial strains (*SI Appendix*, Table S1) were cultured in InsectaGro or MRS broth in the presence or absence of 10 mM glyphosate in a 96-well plate and incubated in a plate reader at 35 °C and 5% CO_2 for 48 h. Optical density was measured at 600 nm every 6 h. Experiments were performed in triplicate.

Plasmid Construction and Transformation. The *aroA*, *yhhS*, and *tetC* genes from various bacterial strains were PCR amplified and cloned into the arabinose-inducible pBAD30 vector (45) by Gibson assembly (46) and then used to transform *E. coli* strain BW25113 or a derivative lacking the *aroA* gene by electroporation. Primer sequences are listed in *SI Appendix*, Table S2.

Growth Rate Analysis of Transformed *E. coli*. Transformed *E. coli* cells were cultured in duplicate in 24-well plates containing M9 minimal medium (47) with appropriate antibiotics, varying concentrations of glyphosate, and varying concentrations of arabinose. The plates were incubated in a plate reader at 37 °C for 24–96 h. Optical density was measured at 600 nm every hour.

ACKNOWLEDGMENTS. We thank Eli Powell and Zack Shaffer for technical assistance, Erik Quandt for cloning advice, Waldan Kwong and Hauke Koch for providing bee gut bacterial strains, Margaret Steele for constructive comments, Kim Hammond for maintaining hives, Jeffrey Barrick for providing *E. coli* Δ*aroA* BW25113, and the UT Austin GSAF for sequencing services. This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (13578-13-8 to E.V.S.M.), the USDA National Institute of Food and Agriculture (2017-67012-26088 to K.R. and 2018-67013-27540 to N.A.M.), and the National Institutes of Health (R01GM108477-02 to N.A.M.).

1. Green JM (2018) The rise and future of glyphosate and glyphosate-resistant crops. *Pest Manag Sci* 74:1035–1039.
2. Van Bruggen AHC, et al. (2018) Environmental and health effects of the herbicide glyphosate. *Sci Total Environ* 616–617:255–268.
3. Shilo T, Zygier L, Rubin B, Wolf S, Eizenberg H (2016) Mechanism of glyphosate control of *Phelipanche aegyptiaca*. *Planta* 244:1095–1107.
4. Funke T, et al. (2009) Structural basis of glyphosate resistance resulting from the double mutation Thr97 -> Ile and Pro101 -> Ser in 5-enolpyruvylshikimate-3-phosphate synthase from *Escherichia coli*. *J Biol Chem* 284:9854–9860.
5. Funke T, Han H, Healy-Fried ML, Fischer M, Schönbrunn E (2006) Molecular basis for the herbicide resistance of Roundup Ready crops. *Proc Natl Acad Sci USA* 103: 13010–13015.
6. Mir R, Jallu S, Singh TP (2015) The shikimate pathway: Review of amino acid sequence, function and three-dimensional structures of the enzymes. *Crit Rev Microbiol* 41: 172–189.
7. Priestman MA, Funke T, Singh IM, Crupper SS, Schönbrunn E (2005) 5-Enolpyruvylshikimate-3-phosphate synthase from *Staphylococcus aureus* is insensitive to glyphosate. *FEBS Lett* 579:728–732.
8. Cao G, et al. (2012) A novel 5-enolpyruvylshikimate-3-phosphate synthase shows high glyphosate tolerance in *Escherichia coli* and tobacco plants. *PLoS One* 7:e38718.
9. Duke SO, Powles SB (2008) Glyphosate: A once-in-a-century herbicide. *Pest Manag Sci* 64:319–325.
10. Balbuena MS, et al. (2015) Effects of sublethal doses of glyphosate on honeybee navigation. *J Exp Biol* 218:2799–2805.
11. Gaupp-Berghausen M, Hofer M, Rewald B, Zaller JG (2015) Glyphosate-based herbicides reduce the activity and reproduction of earthworms and lead to increased soil nutrient concentrations. *Sci Rep* 5:12886.
12. Tsui MTK, Chu LM (2003) Aquatic toxicity of glyphosate-based formulations: Comparison between different organisms and the effects of environmental factors. *Chemosphere* 52:1189–1197.
13. Shehata AA, Schrödl W, Aldin AA, Hafez HM, Krüger M (2013) The effect of glyphosate on potential pathogens and beneficial members of poultry microbiota in vitro. *Curr Microbiol* 66:350–358.
14. Steinrücken HC, Amrhein N (1984) 5-Enolpyruvylshikimate-3-phosphate synthase of Klebsiella pneumoniae 2. Inhibition by glyphosate [N-(phosphonomethyl)glycine]. *Eur J Biochem* 143:351–357.
15. Newman MM, et al. (2016) Glyphosate effects on soil rhizosphere-associated bacterial communities. *Sci Total Environ* 543:155–160.
16. Cariveau DP, Elijah Powell J, Koch H, Winfree R, Moran NA (2014) Variation in gut microbial communities and its association with pathogen infection in wild bumble bees (Bombus). *ISME J* 8:2369–2379.
17. Koch H, Schmid-Hempel P (2011) Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proc Natl Acad Sci USA* 108:19288–19292.
18. Kwong WK, Moran NA (2016) Gut microbial communities of social bees. *Nat Rev Microbiol* 14:374–384.
19. Engel P, Martinson VG, Moran NA (2012) Functional diversity within the simple gut microbiota of the honey bee. *Proc Natl Acad Sci USA* 109:11002–11007.
20. Zheng H, et al. (2016) Metabolism of toxic sugars by strains of the bee gut symbiont *Gilliamella apicola*. *MBio* 7:e01326-16.
21. Powell JE, Martinson VG, Urban-Mead K, Moran NA (2014) Routes of acquisition of the gut microbiota of *Apis mellifera*. *Appl Environ Microbiol* 80:7378–7387.
22. Zheng H, Powell JE, Steele MI, Dietrich C, Moran NA (2017) Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling. *Proc Natl Acad Sci USA* 114:4775–4780.
23. Raymann K, Shaffer Z, Moran NA (2017) Antibiotic exposure perturbs the gut microbiota and elevates mortality in honeybees. *PLoS Biol* 15:e2001861.
24. Herbert LT, Vázquez DE, Arenas A, Farina WM (2014) Effects of field-realistic doses of glyphosate on honeybee appetitive behaviour. *J Exp Biol* 217:3457–3464.
25. Mukherjee I (2009) Determination of pesticide residues in honey samples. *Bull Environ Contam Toxicol* 83:818–821.
26. Thompson HM, et al. (2014) Evaluating exposure and potential effects on honeybee brood (*Apis mellifera*) development using glyphosate as an example. *Integr Environ Assess Manag* 10:463–470.
27. Lozupone C, Knight R (2005) UniFrac: A new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* 71:8228–8235.
28. Li J, et al. (2015) Two gut community enterotypes recur in diverse bumblebee species. *Curr Biol* 25:R652–R653.
29. Moran NA, Hansen AK, Powell JE, Sabree ZL (2012) Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. *PLoS One* 7: e36393.
30. Powell JE, Leonard SP, Kwong WK, Engel P, Moran NA (2016) Genome-wide screen identifies host colonization determinants in a bacterial gut symbiont. *Proc Natl Acad Sci USA* 113:13887–13892.
31. Steele MI, Kwong WK, Whiteley M, Moran NA (2017) Diversification of type VI secretion system toxins reveals ancient antagonism among bee gut microbes. *MBio* 8: e01630-17.
32. Kwong WK, Mancenido AL, Moran NA (2017) Immune system stimulation by the native gut microbiota of honey bees. *R Soc Open Sci* 4:170003.
33. Kwong WK, Moran NA (2016) *Apibacter adventoris* gen. nov., sp. nov., a member of the phylum *Bacteroidetes* isolated from honey bees. *Int J Syst Evol Microbiol* 66: 1323–1329.
34. Corby-Harris V, et al. (2014) Origin and effect of Alpha 2.2 *Acetobacteraceae* in honey bee larvae and description of *Parasaccharibacter apium* gen. nov., sp. nov. *Appl Environ Microbiol* 80:7460–7472.
35. Ebeling J, Knispel H, Hertlein G, Fünfhaus A, Genersch E (2016) Biology of *Paenibacillus larvae*, a deadly pathogen of honey bee larvae. *Appl Microbiol Biotechnol* 100: 7387–7395.
36. Eschenburg S, Healy ML, Priestman MA, Lushington GH, Schönbrunn E (2002) How the mutation glycine96 to alanine confers glyphosate insensitivity to 5-enolpyruvyl shikimate-3-phosphate synthase from *Escherichia coli*. *Planta* 216:129–135.
37. Kešnerová L, et al. (2017) Disentangling metabolic functions of bacteria in the honey bee gut. *PLoS Biol* 15:e2003467.
38. Tian B, Fadhil NH, Powell JE, Kwong WK, Moran NA (2012) Long-term exposure to antibiotics has caused accumulation of resistance determinants in the gut microbiota of honeybees. *MBio* 3:e00377-12.
39. Staub JM, Brand L, Tran M, Kong Y, Rogers SG (2012) Bacterial glyphosate resistance conferred by overexpression of an *E. coli* membrane efflux transporter. *J Ind Microbiol Biotechnol* 39:641–647.
40. Kwong WK, Engel P, Koch H, Moran NA (2014) Genomics and host specialization of honey bee and bumble bee gut symbionts. *Proc Natl Acad Sci USA* 111:11509–11514.
41. Raymann K, Bobay L-M, Moran NA (2018) Antibiotics reduce genetic diversity of core species in the honey bee gut microbiome. *Mol Ecol* 27:2057–2066.
42. Schwarz RS, Moran NA, Evans JD (2016) Early gut colonizers shape parasite susceptibility and microbiota composition in honey bee workers. *Proc Natl Acad Sci USA* 113: 9345–9350.
43. Caporaso JG, et al. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 7:335–336.
44. Martinson VG, Moy J, Moran NA (2012) Establishment of characteristic gut bacteria during development of the honeybee worker. *Appl Environ Microbiol* 78:2830–2840.
45. Guzman L-M, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* 177:4121–4130.
46. Gibson DG, et al. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 6:343–345.
47. Joyce AR, et al. (2006) Experimental and computational assessment of conditionally essential genes in *Escherichia coli*. *J Bacteriol* 188:8259–8271.