Gut bacteria facilitate adaptation to crop rotation in the western corn rootworm

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Insects are constantly adapting to human-driven landscape changes; however, the roles of their gut microbiota in these processes remain largely unknown. The western corn rootworm (WCR, Diabrotica virgifera virgifera LeConte) (Coleoptera: Chrysomelidae) is a major corn pest that has been controlled via annual rotation between corn (Zea mays) and nonhost soybean (Glycine max) in the United States. This practice selected for a “rotation-resistant” variant (RR-WCR) with reduced ovipositional fidelity to cornfields. When in soybean fields, RR-WCRs also exhibit an elevated tolerance of antiherbivore defenses (i.e., cysteine protease inhibitors) expressed in soybean foliage. Here we show that gut bacterial microbiota is an important factor facilitating this corn specialist’s (WCR’s) physiological adaptation to brief soybean herbivory. Comparisons of gut microbiota between RR- and wild-type WCR (WT-WCR) revealed concomitant shifts in bacterial community structure with host adaptation to soybean diets. Antibiotic suppression of gut bacteria significantly reduced RR-WCR tolerance of soybean herbivory to the level of WT-WCR, whereas WT-WCR were unaffected. Our findings demonstrate that gut bacteria help to facilitate rapid adaptation of insects in managed ecosystems.

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by crop rotation. We compared gut-associated bacterial community structures (designated as microbiota structures) of WT- and RR-WCR by incorporating the relative abundance of bacterial operative taxonomic units (OTUs) using automated ribosomal intergenic spacer analysis (ARISA). The functions of WT- and RR-WCR gut microbiotas were also compared by measuring the WCR tolerance of the soybean herbivory and the gut cysteine protease activity of WT- and RR-WCR after antibiotic suppression of gut microbes.

Results

Gut Microbiotas of Field-Collected WCR Populations and Their Correlation with RR/WT Phenotypes. Gut microbiotas are closely linked to insect dietary preferences and trophic interactions (4, 19). To study potential differences between gut microbiotas of WT- and RR-WCR, 16S rDNA clone libraries of gut bacteria of phenotypically representative WT- and RR-WCR [based on their mobility (Fig. S1) and gut physiology (14)] from Higginsville, Missouri (WT) and Urbana, Illinois (RR) were compared. We identified significant proportional changes among five conserved bacterial taxa from Enterobacteriaceae, Lactococcales, and Xanthomona (χ² test, between populations, P < 0.0001), which together represent 70% and 65% of the microbiotas in the WT and RR population, respectively (Fig. 1). In WT-WCR, Enterobacter sp., Lactococcus sp., and Enterococcus sp. together make up 66% of the microbiota, whereas in RR-WCR they represent only 37% of the gut bacteria. In contrast, Klebsiella sp. and Stenotrophomonas sp. represent only 4% of the gut bacteria in WT-WCR, whereas in RR-WCR, the two taxa account for 28% of the gut microbiota. In addition, each population carried unique taxa that are known to exist in the environment (Fig. 1). Substantial compositional/proportional differences between microbiotas of the two populations suggest changes at the community/structural level that may influence gut physiology.

Given the prominent differences between bacterial community structures of the WT- and RR-WCR populations (Fig. 1), we tested whether gut microbiota structures are consistently different between the two phenotypes with multiple WCR populations using ARISA (Fig. 2 A and B). Collected beetles were experimentally kept under dietary conditions that they would encounter in the field (corn, soybean, or starvation) and sampled for their total gut DNA for ARISA (28). The ARISA profiles were compared within a Bray–Curtis dissimilarity matrix incorporating relative abundance of different OTUs (Fig. 2B). Results demonstrated that gut microbiota structures are different between the RR- and WT-WCR (P = 0.0001; Fig. 2B); the effect of dietary treatment (P = 0.0001) and the phenotype–diet interaction were also significant [P = 0.0147; two-way permutational multivariate analysis of variance (PERMANOVA) with Monte Carlo]. There was significantly greater heterogeneity in RR-WCR microbiota structures than in those of WT-WCR (permutational analysis of multivariate dispersions, or PERMDISP, P = 0.0078). When adding “population” as a random factor nested within “phenotype” (three-way nested PERMANOVA), a considerable amount of microbiota structural variation was explained (P = 0.0001). These data indicate correlations of microbiota structures with the RR and WT phenotypes and that there is a high level of heterogeneity in the gut bacterial communities at the population level, especially in RR-WCR.

To investigate correlations of microbiota structures and the RR phenotype at the population level, we extracted ARISA profiles of soybean-fed RR- and WT-WCR for further analysis (soybean was the diet of most interest). Pair-wise PERMANOVA tests showed significant differences in microbiota structures across nearly all populations fed on soybean (Holm–Bonferroni corrected, P < 0.05; with Monte Carlo), with the exception of WT-WCR from Concord, Nebraska, and Boone, Iowa. When analyzed with hierarchical cluster analysis using Ward’s method (29), the samples were divided into three, rather than two, large clusters (Fig. 2C). Cluster I (C-I) is composed of three WT populations, whereas clusters II and III (C-II and -III) are composed of one and two RR populations, respectively. Calculating the geographic distance between Piper City, Illinois—the historical origin and “epicenter” of the RR variant (27)—and each of the collection sites (Table S1) revealed a clustering of microbiota structures that followed the order of each location’s relative distance from the epicenter (Fig. 2C). Crop diversity in the areas where WCR were collected decreased toward the epicenter (Fig. 2C; Table S1). Moreover, the clustering results correlated with the mobility (as measured as time spent to escape a cylindrical arena) of populations from each cluster, with the highest in C-III, followed by C-II, with the lowest mobility in C-I (Fig. S1). Also, there was greater microbial community heterogeneity among geographically clustered RR populations than among relatively dispersed WT populations (Fig. 2), suggesting that the patterns are not artifacts of environmental gradients.

Correlation of WCR Gut Microbiota Structures with WCR Survival on Soybean and Their Gut Cysteine Protease Activity. To test whether the observed gradient in microbiota community dissimilarity corresponded with WCR tolerance of soybean defense, we compared WCR survival (on soybean; Kaplan–Meier survival curves) and gut cysteine protease activities across three representative WCR populations (from each cluster in Fig. 2C). The results confirmed that RR-WCR from clusters II and III experience significantly greater survival on soybean and have higher gut cysteine protease activity than WT-WCR from cluster I (P < 0.05; Figs. 2C and 3), a pattern concordant with previous studies (14). Both phenotypic measurements of each population followed the same order as

![Fig. 1.](image-url)
microbiota structure dissimilarity clustering among soybean-fed WCR (Figs. 2C and 3) and overall population mobility (Fig. S1).

**Contribution of RR and WT-WCR Gut Microbiotas to WCR Survival on Soybean and Their Gut Cysteine Protease Activity.** Using phenotypically well-characterized RR- and WT-WCR populations from Shabbona, Illinois, and Higginsville, Missouri, we compared the survival curves of RR- and WT-WCR adults feeding on soybean foliage following different antibiotic dosages (mixtures of erythromycin, gentamicin, kanamycin, and tetracycline at 0, 50, or 400 mg/L, Fig. 4A and B). After control (0 mg/L) and 50-mg/L antibiotic treatments, the RR-WCR had greater survival on soybeans than WT-WCR ($P < 0.05$). After 400 mg/L treatments, however, there were no significant differences between survivorship of the two phenotypes. Compared with control groups, significant decreases in survivorship ($P < 0.05$) following high-dosage treatments occurred only in RR-WCR and not in WT-WCR (Fig. 4B). In contrast, survival of RR- and WT-WCR on corn diets after the same time period was unaffected by antibiotic treatments compared with control groups (Fig. S2A).

Because high activity levels of cysteine proteases in RR-WCR guts were previously demonstrated to explain their prolonged survival on soybean (14), we measured those levels in the two WCR phenotypes (populations from Urbana, IL, and Higginsville, MO) following the antibiotic treatments described above. Treatment with 400 mg/L of antibiotics significantly lowered the protease activity of RR-WCR to the level of WT-WCR (Fig. 4C). In the WT-WCR, protease activities were unaffected by antibiotic treatment (Fig. 4C).

To test whether reduced protease activity in RR-WCR after the 400-mg/L antibiotic treatments was related to antibiotic effects on WCR protease gene expression, the expression of cysteine protease gene DrRS5 (GenBank accession no. AJ583508) (30)—the most highly expressed protease gene in WCR when feeding on soybean foliage (14)—was measured in RR- and WT-WCR treated with 400 mg/L of antibiotics or water (control groups; Fig. S2B). Antibiotic treatments had virtually no effect on DrRS5 expression in both WCR phenotypes ($F = 0.076$, df = 1, $P = 0.79$), supporting the idea that gut bacteria are the main cause of the phenotypic differences among RR-WCR that received different antibiotic treatments (Fig. 4C).

**Discussion**

Human-mediated landscape changes are inducing insect adaptation to ecological disturbances at an unprecedented scale and pace (31). We have demonstrated that within few decades crop rotation has selected for a distinctly altered microbiota in RR-WCR, which provides digestive advantages endowing RR-WCR with enhanced tolerance of soybean defenses. Although it is unclear whether tolerance of soybean defense enabled the RR-WCR to reduce their fidelity to corn or was a subsequent
adaptation following relaxation of host fidelity, improved RR-WCR performance in a nonhost environment led to greater reproductive success in rotated corn and soybean ecosystems. These changes present a mechanism facilitating WCR adaptation to cultural control that could lead to further ecological divergence if human-driven selection continues.

The gut bacterial community structures of RR-WCR populations were different from those of WT-WCR populations (Fig. 2B). The scale of dissimilarities between the microbiota structures of soybean-fed WCR paralleled their distances from the historical epicenter of rotation resistance, their mobility (Fig. S1) and capability to tolerate soybean diets, and the landscape heterogeneity of the area where they were collected (Figs. 2C and 3). Mobility assays showed proportional differences in adult mobility among and within RR-WCR populations (Fig. S1). In addition, previous studies indicated that movement into soybean fields and subsequent soybean herbivory, although rare, occurs in WT-WCR populations. It is the proportion of beetles exhibiting these behaviors that have greatly increased in RR-WCR populations (6). Also, a role for landscape heterogeneity in the selection of higher tolerance of soybean diets in RR-WCR has been proposed (14). Our data therefore suggest that the proportion of RR-WCR harboring distinctive gut microbiota within populations is distributed in a gradient reflecting the penetration of RR phenotypes into various parts of the Corn Belt. Gut microbiotas are known to regulate or contribute to insect digestive enzyme activities (25, 26). We demonstrated that the RR-WCR microbiota contribute to the proteolysis and survival of the WCR on soybeans (Fig. 4). These results, together with the RR-WCR’s digestive adaptation to soybean CystPIs, (14) suggest that the functionally distinctive RR-WCR gut microbiota could act as an adaptive trait that persists among WCR in rotated corn and soybean agroecosystems.

Our study of gut bacterial clone libraries revealed substantial differences between conserved/unique bacterial taxa in WT- and RR-WCR guts (Fig. 1). Various bacterial species could produce intra/extracellular proteases (32–35), regulate host gene expression in the gut (36, 37), or modify biochemical properties of their surrounding environments (such as Enterococcus species in termitines) (38). Moreover, species like Lactococcus sp. are known to regulate the growth of other gut bacteria (39, 40), which suggests correlations between their relative abundance and the gut bacterial community structure. These interactions involve complex mechanisms that are difficult to dissect based on the identity of individual taxa that are mostly environmental bacteria. The RR phenomenon itself is also intrinsically obscured because genetic diagnostic markers differentiating RR individuals from WT are lacking (41). Moreover, modeling studies showed that the spread of the RR phenotype could be explained by the expansion of an adaptive allele across populations rather than by displacement of WT-WCR by a “RR strain” (42), indicating that heterogeneity in allele frequency of the gene(s) responsible can exist in any RR-WCR population. Given these challenges, we considered the host (WCR), gut bacterial microbiota, and environment as entities and studied their interconnections. By comparing quantitative measurements representing these components at the population level, we demonstrated that the microbiotas are not merely passive players influenced by the host, but functional components of an insect mechanism to confront dietary stress. This adaptation could affect other aspects of insect biology, such as distorting the outcomes of a pest’s reduced fidelity to optimal diets. Recognizing host–microbiota interactions as potent ecological factors facilitating insect resistance evolution may provide avenues for pest resistance management and for developing pest control strategies.

Materials and Methods

Insect and Plant Materials. Insects were collected and bioassayed from July to August in 2010–2012. WT-WCR adults were collected as individuals from cornfields in Concord, Nebraska; Higginsville, Missouri; and Boone, Iowa, whereas RR-WCRs were collected from Urbana, Illinois; Minonk, Illinois; and Shabbona, Illinois (Fig. 2A and Table S1). Sampling sites are separated by over 100 km from any other site so that confounding effects of local adaptation did not restrict the analyses. Populations were separated into different phenotypes based on the documented RR-WCR distribution (7). Four populations (all except Boone, IA, and Shabbona, IL) have been phenotypically characterized in previous work (14). Moreover, four of the most abundant populations (all except Boone, IA, and Minonk, IL) were tested for their mobility; each population included some individuals exhibiting characteristics of WT-WCR (longer escape time — lower mobility) and RR-WCR populations (shorter escape time — greater mobility) (Fig. S1). Collection of all field populations was completed within a week to reduce insect phenology effects. To minimize laboratory effects on gut physiology and microbiota composition, WCR were maintained on corn ears from their field of collection; WCR were used in experiments soon after collection. Correlation of gut microbiota structures with the RR phenotype was first tested across all populations. Thereafter, because of limited insect availability, phenotypically representative populations of WT- and RR-WCR were used to conduct subsequent bioassays and antibiotic treatments.

Soybean plants (G. max "Williams 82") were grown in a greenhouse under light intensities of 1,200–1,500 μmol·m⁻²·s⁻¹ at the University of Illinois at Urbana–Champaign for 28 d. For dietary treatments, corn ears (Z. mays "Sugar Buns") grown in a greenhouse and prepared from the same batch of hand-harvested corn.

Constructing and Sequencing 16S rDNA Libraries of WCR Gut Microbota. Gut DNA from Urbana, Illinois, and Higginsville, Missouri, WCR beetles feeding on a soybean diet (Williams 82) for 48 h were separately used as templates.

**Fig. 4.** Effects of different antibiotic dosage treatments (0, 50, or 400 mg/L) on gut microbial growth and soybean tolerance in WT- and RR-WCR. (A) Antibiotic treatments (dosage indicated on the left) suppressed growth of culturable bacteria in WT- and RR-WCR guts. Pulverized gut-tissue suspensions (104-fold diluted, 100 μL) were dropped on nutrient agar plates and cultured for 48 h at 30 °C. (B) Survival of WT-WCR (Higginsville, MO, population) and RR-WCR (Shabbona, IL, population) treated with different antibiotic concentrations (mg/L) before feeding on soybean plants. Significant differences (log-rank test, Tukey–Kramer adjusted) between survival curves were declared at P < 0.05 (letters next to the key). Crosses represent WCRs treated with 400 mg/L of antibiotics and fed with corn (CR) during the same experiment. (C) Effects of antibiotic treatments on gut cysteine protease activities in WT-WCR (Higginsville, MO, population) and RR-WCR (Urbana, IL, population). Protease activities are expressed as nanomoles of pNA released per milligram of gut protein per minute. Different letters indicate significant differences between treatments (Fisher’s LSD; P < 0.05).
(100 ng) for PCR using bacterial universal primers (Table S1). For each population, total gut DNA was extracted from a pool of 20 WCR guts (10 per sex). Amplified products including bacterial 16S rDNA were cloned into the pGEM-T Easy vector (Promega Inc.). Inserts were sequenced using primers 27f and 1525r (43). Details of clone screening (Fig. S3) and data processing are described in SI Materials and Methods.

Comparison of WCR Gut Microbiota Structures Under Different Dietary Treatments. Field-collected WCR populations were kept separately on soybean, corn, or a starvation treatment for 48 h and asexually dissected to collect their complete digestive tracts (SI Materials and Methods). Bacterial community profiles incorporating relative abundance of bacterial OTUs were analyzed and compared across populations and treatments. For DNA sampling, 20 WCR guts (10 per sex) were pooled as one biological replicate and subjected to DNA extraction using the FastDNA SPIN kit for soil (MP Biomedicals). Three to four samples were collected for each population diet combination. In microbial community analyses using ARISA, 69 gut DNA samples (from a total of 1,380 WCR adults) were used as templates for amplification using the primers ITSf and ITSReub (44), with the former 5′-labeled with the fluorescent dye 6-carboxyfluorescein (SI Materials and Methods). Amplified products were analyzed using the ABI 3730xl genetic analyzers (Applied Biosystems Inc.). Details of ARISA and data processing are presented in SI Materials and Methods.

Multivariate Statistical Methods and Environmental Data. Using Bray–Curtis dissimilarity measures calculated from analyzed ARISA profiles, nonmetric multidimensional scaling, PERMANOVA, PERMDISP (PRIMER6, PRIMER-E Ltd.), and hierarchical cluster analysis (SAS 9.2, SAS Institute Inc.) were conducted to determine data structures and associations of WCR microbiota with diet and phylo- or genotype, population, and diets (SI Materials and Methods). To estimate the cropping diversity of each WCR sampling site, county-scale landscape heterogeneity values (percentage of land area that is neither corn nor soybean, obtained from online databases) were calculated for each site (Table S1).

WCR Survival Test. Tests of field-collected WCR survival on soybean plants were conducted as previously described (14) with slight changes (SI Materials and Methods). Before the tests, 30–35 female WCR from different treatments were starved for 36 h (with water) to facilitate soybean herbivory. Soybean plants were pretreated with 250 μM of methyl-jasmonate 4 d before the tests as described in a previous study for induction of soybean CystPs (15). Survival data were analyzed using the Kaplan–Meier method (45). Survival distribution curves of all treatment groups (phenotype × antibiotic treatment) were compared across populations and treatments. Survival of WT-WCR (Higginsville, MO) and RR-WCR (Shabbona, IL) treated with 400 mg/L of antibiotics were also compared with those from control groups (water only) or corn diets for 6 d. All antibiotic-treated insects were then subjected to survival and protease activity tests described above. Antibiotic-treated samples were also used for total RNA extraction and determination of DvRSS expression using the primers DvRSS-rf/rf and EF-rt/rf (internal control; Table S2).

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Determination of Gut Cysteine Protease Activities in WCR. Before gut sampling, WCR were either standardized on identical corn diets for 48 h (for comparisons across populations) or subjected to antibiotic treatments on the same commercial diets (described below). Independent triplicates of gut samples pooled from multiple insects of equal sex ratios (10 for antibiotic treatment tests and 6 for comparison across populations) were extracted for their gut proteases (SI Materials and Methods). After inhibition of indistinguishable cathepsin B activity by the inhibitor CA-074 (L-3-trans-propylcarbamoyl oxirane-2-carboxylic)-1-isoleucyl-3-proline (14), constitutive cysteine protease activities of each sample were determined by monitoring substrate (L-lysoprolutamyl-L-phenylalanyl-L-leucine-p-nitroanilide (p-Glu-Phe-Leu-PNa)) cleavage at 405 nm using a spectrophotometer (SI Materials and Methods). The Bradford method (Bio-Rad Laboratories Inc.) was used to determine protein concentrations of all protease samples. Protease activity measurements were then calculated into units of nanomoles of protein per minute and compared across treatments using one-way ANOVA.

Antibiotic Treatment of WCR adults. To suppress gut microbes, antibiotic mixtures of erythromycin, gentamicin, kanamycin, and tetracycline were added to sterile water and flash-automated commercial diets (BioS erv; FY7668) to achieve low (50 mg/L) or high (400 mg/L) concentrations and fed to the insect, feeding, control groups, antibiotic solutions were replaced with sterile water. Before treatments, WCR were starved for 12 h to promote diet ingestion. Each treatment was replicated in three containers and continued for 5 d. To confirm treatment effectiveness, guts from three treated insects were pooled and pulverized in 100 μL of sterile water. After 10-fold dilution, 100 μL of these suspensions was dropped on nutrient agar and grown for 48 h at 30 °C (Fig. 4A). To rule out detrimental effects (to the WCR) caused by antibiotics, survival of WT-WCR (Higginsville, MO) and RR-WCR (Shabbona, IL) treated with 400 mg/L of antibiotics were also compared with those from control groups (water only) or corn diets for 6 d. All antibiotic-treated insects were then subjected to survival and protease activity tests described above. Antibiotic-treated samples were also used for total RNA extraction and determination of DvRSS expression using the primers DvRSS-rf/rf and EF-rt/rf (Fig. S4A). To confirm treatment effectiveness, guts from three treated insects were pooled and pulverized in 100 μL of sterile water. After 10-fold dilution, 100 μL of these suspensions was dropped on nutrient agar and grown for 48 h at 30 °C (Fig. 4A). To rule out detrimental effects (to the WCR) caused by antibiotics, survival of WT-WCR (Higginsville, MO) and RR-WCR (Shabbona, IL) treated with 400 mg/L of antibiotics were also compared with those from control groups (water only) or corn diets for 6 d. All antibiotic-treated insects were then subjected to survival and protease activity tests described above. Antibiotic-treated samples were also used for total RNA extraction and determination of DvRSS expression using the primers DvRSS-rf/rf and EF-rt/rf (internal control; Table S2).