Supporting Information

Chu et al. 10.1073/pnas.1301886110

SI Materials and Methods

Western Corn Rootworm Beetle Mobility Test. Mobility measurements of each western corn rootworm (WCR) population were conducted using previously described methods (1) with slight modification. WCR females from four populations (all except the Boone, IA, and Minonk, IL, populations, which were available in limited insect numbers) were standardized on the same corn diets for 48 h. Individual beetles were then placed into the bottom of a 17-cm diameter × 45-cm tall cylinder with a 10-cm tall conical screen top (the arena) and measured for the time it spent to escape from the top. Beetles that took more than 120 s to escape were removed from the arena and censored in the following analysis. Analyses were conducted using the log-rank test (LIFETEST procedure, Tukey–Kramer adjusted) in SAS.

Sampling of Total Gut DNA. WCR under different treatments (population × diet combination) were anesthetized with chloroform and surface-sterilized in 70% (vol/vol) alcohol for 30 s. Individuals were then washed with sterile 0.1% (vol/vol) Triton-X-100 for 5 min and rinsed twice in distilled water to remove possible surface contaminants. Insect digestive tracts were excised in sterile Ringer's solution (0.75 g NaCl, 0.35 g KCl, 0.28 g $CaCl_2$ in 1 L of distilled water) (2) and extracted for their DNA with the FastDNA SPIN kit for soil (MP Biomedicals) following the supplier's instructions.

Constructing and Sequencing 16S rDNA Libraries of WCR Gut Microbiotas. Sequenced data were screened for chimeras using Mallard 1.02 (3). The data were aligned (SILVA reference alignment) and binned with the Mothur software (4) at 98% threshold of sequence similarity. Taxa [operative taxonomic units (OTUs)] that appeared only once in one of the two populations were considered noise and filtered (only two taxa were considered noises). In total, 154 and 142 clones were screened for the Urbana, Illinois (UIL) and Higginsville, Missouri (HMO) population, respectively (Fig. S3). The proportion of each taxon in each WCR population was calculated. The top BLAST hit for each OTU was reported to the genus level. Sequences have been deposited in GenBank under accession nos. KC865711–KC865726.

Details of Automated Ribosomal Intergenic Spacer Analysis and Data Processing. Amplification of bacterial intergenic spacer regions by PCR was conducted with 100 ng of template DNA using the primer pair ITSF and ITSReub (Table S2) (5), with the former 5′ labeled with the phosphoramidite dye 6-carboxyfluorescein (6-FAM). Conditions for PCR were set at 94 °C for 2 min, followed by 30 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. Amplified products were analyzed using capillary electrophoresis in the ABI 3730xl genetic analyzers. Abundance measures of each OTU (nucleotide fragment of unique length produced in the PCR) represented by peak areas were identified using the Genemapper software (Applied Biosystems, Inc.). All OTUs below 200 bp and over 1 kb were excluded from the analysis to assure the accuracy of fragment size calling. Noise filtering and OTU binning procedures were completed within the T-REX software (6). A blank reaction without template DNA was analyzed, and the largest noise signal (peak height) was used to set a filtering threshold. The OTU binning was conducted with a clustering threshold of 1.2 bp. All OTUs with single occurrence among biological replicates were also excluded at this step to reduce the influences of outliers. To account for run-to-run variation, Hellinger-transformed data (7) were used to calculate Bray–Curtis dissimilarity measures representing the relationships across microbiota diversities using the "vegan" package in R (8).

Multivariate Statistics. Nonmetric multidimensional scaling (nMDS) was used to visualize the microbiota data and to explore factors explaining relationships between observations. Using Bray–Curtis dissimilarity measures calculated as described in the previous section, an nMDS solution with a Kruskal's Stress (formula I) value of 0.16 was obtained and plotted in 3D space using PRIMER6 (PRIMER-E Ltd.) and SPSS (IBM SPSSInc.). Two permutational multivariate analysis of variance (PERMANOVA) (9) tests were conducted to examine factor significances affecting WCR gut microbiota structure. A two-way (phenotype × diet) or three-way model (additional random factor "population" nested in the "phenotype") was applied (type III sums of squares, permutations of residuals under a reduced model, 9999 permutations) with Monte Carlo permutation testing. Comparison of multivariate dispersion between phenotypes was analyzed by permutational analysis of multivariate dispersions (PERMDISP) (10) using 9999 permutations. To uncover relationships among WCR microbiota structures at the population level, a Bray–Curtis dissimilarity matrix calculated from automated ribosomal intergenic spacer analysis (ARISA) profiles of WCR fed on a soybean diet was analyzed with single-factor (population) pairwise PERMANOVA and Holm–Bonferroni correction (11). In addition, the dissimilarity matrix was used for hierarchical cluster analysis (Ward's method) (12) in SAS (SAS Institute Inc.). An additional analysis using the group average method was also conducted and confirmed to produce the same clustering results.

Survivorship Tests of WCR on Soybean Plants. Insects were separately placed in 30-cm diameter pots containing four 28-d-old soybean plants. Before insect introduction, the soil surface of each pot was covered with aluminum foil, and the pot was enclosed within a clean mesh bag to prevent insect escape. All experiments were conducted in growth chambers set at 24 °C, 70–90% relative humidity (RH), and 14:10 light:dark photoperiod. Dead insects were counted and removed daily.

Determination of Gut Cysteine Protease Activities in WCR. For protease extraction, gut samples were homogenized in 30 mM of Tri-K citrate (pH 6.0, 2.5 μL/mg gut tissue) using micropestles and incubated on ice for 30 min. After 15 min of centrifugation at 12,000 × *g*, supernatants (1 μL) from each sample were added to 79 μL of reaction buffer (0.1 M NaH_2PO_4, 0.3 M KCl, 0.1 mM EDTA, and 3 mM DTT at pH 6.0) containing 1 μM of cathepsin B inhibitor CA-074 [L-3-trans(propylcarbamyl) oxirane-2-carbonyl)-L-isoleucyl-L-proline] (13) and incubated for 10 min at 37 °C. Inhibition of cathepsin B proteases (inhibitor-inducible proteases) was conducted to allow accurate measurements of constitutive cysteine protease activities (14). Proteolysis reactions were started by adding the synthetic protease substrate L-pyroglutamyl-L-phenylalanyl-L-leucine-p-nitroanilide (p-Glu-Phe-Leu-pNA) to a final concentration of 76 μM. During the reaction, pNA released by substrate hydrolysis was measured with a spectrophotometer (ELx808, Bio-Tek Inc.) at 405 nm.

Extraction of RNA and Quantitative RT-PCR. Three biological replicates ($n = 3$) of gut samples (each pooled with guts from two males and three females) were collected for each treatment group (phenotype × antibiotic dosage). Total RNAs were extracted from collected WCR gut tissues using the E.Z.N.A Total RNA Kit I

(Omega Bio-Tek Inc.) including the optional DNase treatments. Extracted RNAs (250 ng) were reverse-transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen Inc.) with oligo(dT) primers. Ten-fold diluted cDNA products were used as templates for quantitative RT-PCR (qRT-PCR) analysis (SYBR green method) with an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems Inc.) and the GoTaq qPCR Master Mix (Promega Inc.). For each biological replicate, two technical replicates were used. The PCR conditions were set at 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 58 °C for 1 min, following a dissociation stage.

Before analysis, PCR efficiencies of primer pairs of each gene were determined, reaching 93.87% (cysteine protease gene *DvRS5*) and 91.94% (elongation factor gene *EF-1α*) (both with $R^2 > 0.998$). Both primer pairs produced single amplicons under the PCR condition above in standard curve assays. The PCR products of primer pairs (Table S2) specific to the cathepsin L gene *DvRS5* or the internal control *EF-1α* were confirmed to be correct targets. After gene expression measurements, data were analyzed using the $\Delta\Delta C_T$ method (15). Expression of *DvRS5* was normalized against *EF-1α* within each biological replicate (ΔC_T). Expression levels relative to that of wild type (WT)-WCR receiving the control treatment (0 mg/L) were calculated for each treatment group ($\Delta\Delta C_T$). The relative expression levels were compared across treatment groups with a two-way ANOVA (phenotype × antibiotic dosage). Data were exponentially transformed to fold changes in transcript abundance for figure illustration.

1. Knolhoff LM, Onstad DW, Spencer JL, Levine E (2006) Behavioral differences between rotation-resistant and wild-type *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae). *Environ Entomol* 35(4):1049–1057.
2. Lundgren JG, Lehman RM, Chee-Sanford J (2007) Bacterial communities within digestive tracts of ground beetles (Coleoptera: Carabidae). *Ann Entomol Soc Am* 100(2):275–282.
3. Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ, Weightman AJ (2006) New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Appl Environ Microbiol* 72(9):5734–5741.
4. Schloss PD, et al. (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 75(23):7537–7541.
5. Cardinale M, et al. (2004) Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. *Appl Environ Microbiol* 70(10):6147–6156.
6. Culman SW, Bukowski R, Gauch HG, Cadillo-Quiroz H, Buckley DH (2009) T-REX: Software for the processing and analysis of T-RFLP data. *BMC Bioinformatics* 10:171.
7. Legendre P, Gallagher ED (2001) Ecologically meaningful transformations for ordination of species. *Oecologia* 129(2):271–280.
8. Oksanen J, et al. (2008) Vegan: Community Ecology Package, R package Version 1.15-1. Available at http://vegan.r-forge.r-project.org/.
9. Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecol* 26(1):32–46.
10. Anderson MJ (2006) Distance-based tests for homogeneity of multivariate dispersions. *Biometrics* 62(1):245–253.
11. Holm S (1979) A simple sequentially rejective multiple test procedure. *Scand J Stat* 6(2):65–70.
12. Ward JH (1963) Hierarchical grouping to optimize an objective function. *J Am Stat Assoc* 58(301):236–244.
13. Murata M, et al. (1991) Novel epoxysuccinyl peptides. Selective inhibitors of cathepsin B, *in vitro*. *FEBS Lett* 280(2):307–310.
14. Curzi MJ, Zavala JA, Spencer JL, Seufferheld MJ (2012) Abnormally high digestive enzyme activity and gene expression explain the contemporary evolution of a *Diabrotica* biotype able to feed on soybeans. *Ecol Evol* 2(8):2005–2017.
15. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2(^{-\Delta\Delta}C_{(T)})$ Method. *Methods* 25(4):402–408.

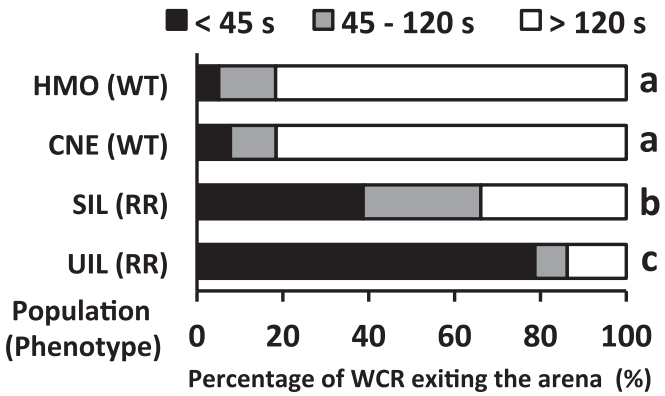

Fig. S1. Comparison of WCR beetle mobility in different WT- and RR-WCR populations using methods modified from those described by Knolhoff et al. (1) (*SI Materials and Methods*). The time required for each WCR beetle standardized on the same corn diet to escape an arena (composed of a cylinder and a screen cone with an exit hole at the top) was measured. Percentages of insects escaping within specified time intervals are indicated by different shading. A total of 80, 124, 103, and 98 individuals were tested for the UIL (Urbana, IL), SIL (Shabbona, IL), CNE (Concord, NE), and HMO (Higginsville, MO) populations, respectively. Mobility time data were analyzed using the log-rank test (Tukey–Kramer adjusted). Significant differences between measurements of each population were declared at $P < 0.05$ (letters on the right of each bar).

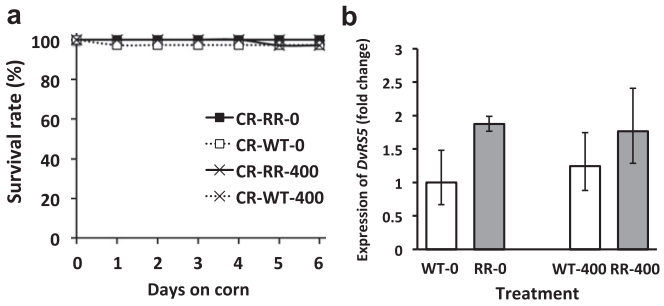

Fig. S2. (*A*) Survival of rotation-resistant (RR)-WCR and WT-WCR on corn diets (CR) was unaffected by antibiotic treatments (400 mg/L dosage; described in *Materials and Methods*) compared with control groups (0 mg/L dosage, water only). (*B*) Expression of cathepsin L gene *DvRS5* in digestive tracts of WT-WCR (Higginsville, MO) and RR-WCR (Urbana, IL) treated with antibiotics (400 mg/L) or water (0 mg/L). Expression data were analyzed using the $\Delta\Delta C_T$ method. Data were exponentially transformed to fold changes in transcript abundance for figure illustration. The error bars represent SEs ($n = 3$). A two-way ANOVA (phenotype × antibiotic dosage) revealed that effects of phenotype ($F = 2.867$, df = 1, $P = 0.129$), antibiotic dosage ($F = 0.076$, df = 1, $P = 0.79$), and their interaction ($F = 0.232$, df = 1, $P = 0.643$) were statistically insignificant.

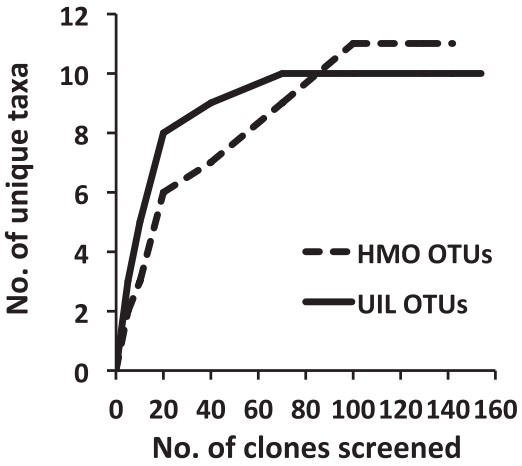

Fig. S3. Collector's curves of 16S rDNA clone libraries constructed from WT-WCR (HMO, Higginsville, MO) and RR-WCR (UIL, Urbana, IL) gut microbiotas.

Table S1. Western corn rootworm collection sites, their distance from the reported epicenter of the RR phenotype, and the county-scale landscape heterogeneity (cropping diversity) around sampling sites

Location	Coordinate	Rotation-resistant status*	County land (ha) (1)	Corn planted (ha) (2)	Soybean planted (ha) (2)	County land area other than corn or soybean, %	Distance to Piper City, IL
Piper City (Ford County), IL	40°45′24″N, 88°11′28″W	RR (epicenter)	125,775	64,750	47,753	11	0 km
Urbana (Champaign County), IL	40°09′14″N, 88°08′40″W	RR	258,033	124,036	91,864	16	67.1 km
Minonk (Woodford County), IL	40°51′26″N, 89°00′26″W	RR	136,700	60,703	39,255	27	69.8 km
Shabbona (DeKalb County), IL	41°50′36″N, 88°50′58″W	RR	163,509	99,148	33,589	19	132.7 km
Boone (Boone County), IA	42°00′31″N, 93°47′19″W	WT	148,036	68,594	40,873	26	488.3 km
Higginsville (Lafayette County), MO	39°07′09″N 93°49′42″W	WT	162,763	39,578	44,920	48	514.9 km
Concord (Dixon County), NE	42°23′39″N, 96°57′23″W	WT	123,343	49,048	32,092	34	752.9 km

*RR, rotation-resistant; WT, wild-type.

1. United States Census Bureau (2010) USA Counties Data. Available at http://censtats.census.gov/.
2. United States Department of Agriculture/National Agricultural Statistics Service (2011) Quick Stats 2.0. Available at http://quickstats.nass.usda.gov/.

Table S2. Sequence and description of primers used in the present work

Primer name	Sequence (starting with 5′)	Description
ITSF (6-FAM at 5′)	GTCGTAACAAGGTAGCCGTA	Forward primer for bacterial ARISA
ITSReub	GCCAAGGCATCCACC	Reverse primer for bacterial ARISA and 16S rDNA clone library construction
27f	AGAGTTTGATCCTGGCTCAG	Construction and sequencing of 16S rDNA clone libraries
1525r	TAAGGAGGTGATCCAGCC	Sequencing of 16S rDNA clone library
DvRS5-rtF	AACGCCATGGTCTCTCTTCCGAAT	Forward primer for qRT-PCR of WCR *DvRS5*
DvRS5-rtR	TGGAAACTGGACCTACGCTAGCAA	Reverse primer for qRT-PCR of WCR *DvRS5*
EF-rtF	TGGACTCGACTGAACCAGCATACA	Forward primer for qRT-PCR targeting WCR *EF-1*α
EF-rtR	ATGTTGTCTCCGTGCCATCCTGAA	Reverse primer for qRT-PCR targeting WCR *EF-1*α