Symbiont-mediated insecticide resistance

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Development of insecticide resistance has been a serious concern worldwide, whose mechanisms have been attributed to evolutionary changes in pest insect genomes such as alteration of drug target sites, up-regulation of degrading enzymes, and enhancement of drug excretion. Here, we report a previously unknown mechanism of insecticide resistance: Infection with an insecticide-degrading bacterial symbiont immediately establishes insecticide resistance in pest insects. The bean bug *Riptortus pedestris* and allied stinkbugs harbor mutualistic gut symbiotic bacteria of the genus *Burkholderia*, which are acquired by nymphal insects from environmental soil every generation. In agricultural fields, fenitrothion-degrading *Burkolderia* strains are present at very low densities. We demonstrated that the fenitrothion-degrading *Burkholderia* strains establish a specific and beneficial symbiosis with the stinkbugs and confer a resistance of the host insects against fenitrothion. Experimental applications of fenitrothion to field soils drastically enriched fenitrothion-degrading bacteria from undetectable levels to >80% of total culturable bacterial counts in the field soils, and >90% of stinkbugs reared with the enriched soil established symbiosis with fenitrothion-degrading *Burkholderia*. In a Japanese island where fenitrothion has been constantly applied to sugarcane fields, we identified a stinkbug population wherein the insects live on sugarcane and ≈8% of them host fenitrothion-degrading *Burkholderia*. Our finding suggests the possibility that the symbiont-mediated insecticide resistance may develop even in the absence of pest insects, quickly establish within a single insect generation, and potentially move around horizontally between different pest insects and other organisms.

pesticide | biodegradation | gut symbiosis | mutualism

Chemical insecticides are used worldwide for controlling agricultural, medical, and hygienic pest insects and other organisms, which have greatly contributed to world's agriculture, economy, and public health. Meanwhile, insecticide abuse has often led to the development of insecticide resistance in diverse pest organisms. Mechanisms underlying the insecticide resistance may involve alteration of drug target sites, up-regulation of degrading enzymes, and enhancement of drug excretion, which are generally attributable to mutational changes in the pest insect genomes (1–3).

Fenitrothion, or *O,O*-dimethyl *O*-(4-nitro-*m*-tolyl) phosphorothioate (Fig. 1*A*), is one of the most popular organophosphorus insecticides used worldwide, which inhibits arthropod acetylcholine esterases and exhibits both oral and percutaneous arthropod-specific toxicities (4). Intensive insecticide applications often result in accelerated biodegradation of the insecticide in the environment (5–7). Previous studies showed that repeated applications of fenitrothion cause drastic increase of fenitrothion-degrading *Pseudomonas*, *Flavobacterium*, and *Burkholderia* in agricultural field soils (8–10). These bacteria are able to hydrolyze fenitrothion into 3-methyl-4-nitrophenol with little insecticidal activity and metabolize the degradation product as a carbon source for their growth (Fig. 1*A*).

The bean bug *Riptortus pedestris* (Fig. 1*B*), known as a notorious pest of leguminous crops (11), is associated with gut bacterial symbiont of the genus *Burkholderia* in a posterior region of the midgut (12) (Fig. 1 *C–E*). In the specialized symbiotic organ, an adult insect harbors as many as 10^8 cells of the *Burkholderia* symbiont, which is culturable on standard microbiological media (13). The symbiont infection enhances growth and size of the host insect, indicating beneficial nature of the symbiosis (13). In contrast to vertical symbiont transmission ubiquitously found in many insects (14, 15), *R. pedertris* environmentally acquires the *Burkholderia* symbiont at the second instar stage from the soil (16), which is similar to environmental acquisition of *Rhizobium* by leguminous plants. Recent extensive surveys revealed that a number of stinkbug species allied to *R. pedestris* are also associated with the *Burkholderia* gut symbionts (17). The stinkbug–*Burkholderia* associations are promiscuous both evolutionarily and ecologically: The symbiont phylogeny does not reflect the host phylogeny, and the stinkbug-associated symbiont strains form a mixed clade with soil-derived *Burkholderia* isolates (12, 13, 17). Notably, the symbiont clade contains a number of soil-derived, fenitrothion-degrading *Burkholderia* isolates (Fig. 1 *F* and *G*), which prompted us to examine the possibility as to whether the fenitrothion-degrading *Burkholderia* strains could establish symbiotic association with these stinkbugs and could confer fenitrothion resistance on the host insects.

Results and Discussion

Fenitrothion-Degrading *Burkholderia* Strains Can Establish Symbiotic Association with *R. pedestris*. We selected three pairs of fenitrothion-degrading environmental *Burkholderia* strains and non-degrading symbiotic *Burkholderia* strains, SFA1 and RPE67, KM-A and RPE301, and KM-G and RPE239, which are closely related to each other genetically but distinct in their origin and fenitrothion-degrading capability (Fig. 1 *F* and *G*, Fig. S1, and Tables S1 and S2). When each of the *Burklolderia* strains was orally administrated to second instar nymphs of *R. pedestris*, not only the symbiotic strains but also the fenitrothion-degrading strains established specific infection to the posterior midgut (Fig. 2 *A–F*). The symbiont titers in the symbiotic organ were at similar levels in the KM-A/RPE301 pair, whereas the symbiotic strains exhibited higher infection titers than the corresponding fenitrothion-degrading strains in the SFA1/RPE67 and KM-G/RPE239 pairs (Fig. S2*A*). Strikingly, not only the insects infected with the symbiotic *Burkholderia* strains but also the insects infected with the fenitrothion-degrading *Burkholderia* strains exhibited higher survival rate, shorter nymphal period, and larger body size than uninfected insects (Fig. S2 *B–G*). Meanwhile, fitness effects of the fenitrothion-degrading *Burkholderia* strains

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB232333, AB621697–AB621699, AB621702–AB621704, AB622644, AB622646, AB622648–AB622682, and AB665358–AB665363).

See Commentary on page 8364.

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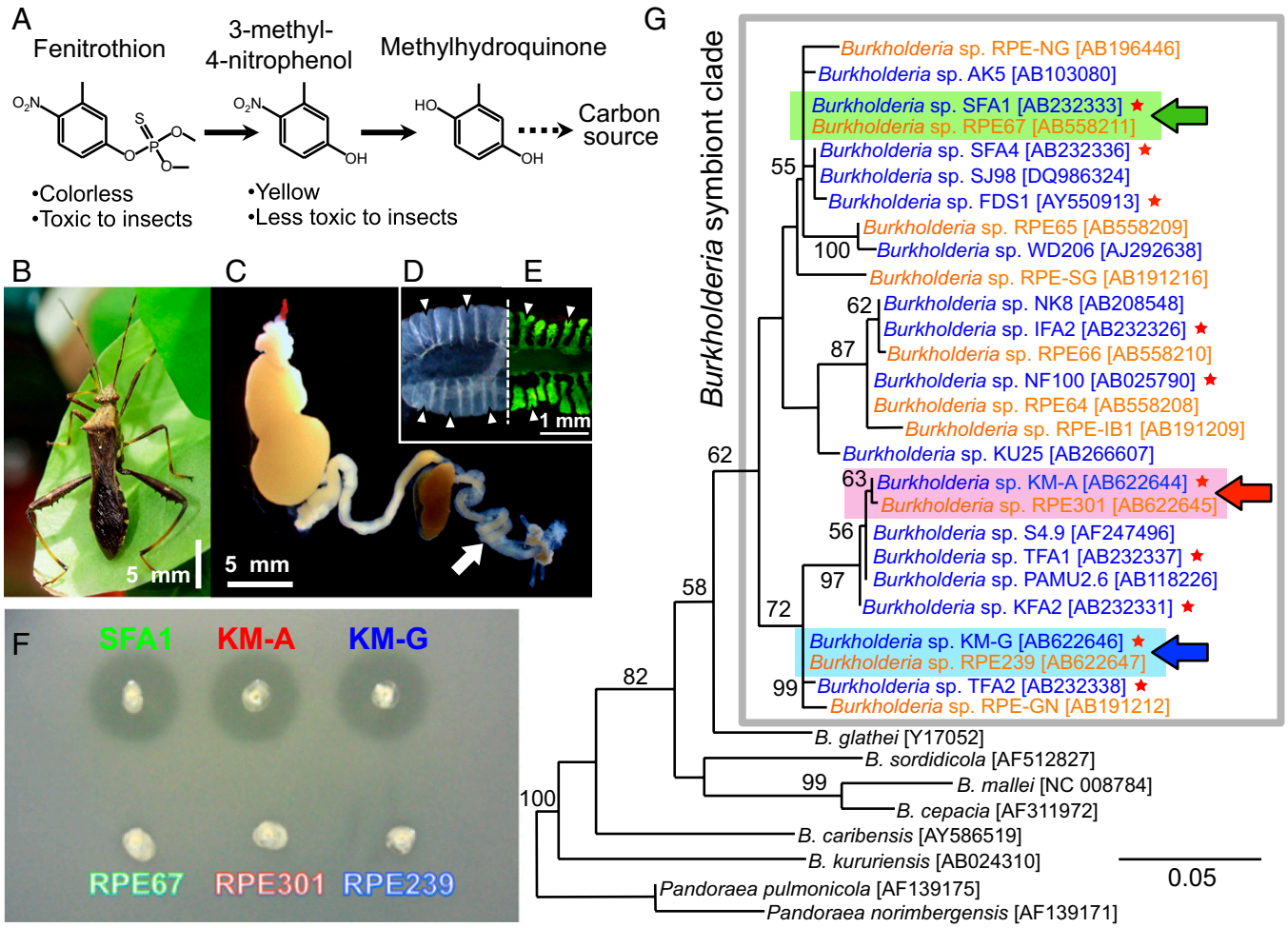

Fig. 1. Fenitrothion degradation by *Burkholderia*, and *Burkholderia* gut symbiont of *R. pedestris*. (*A*) Fenitrothion degradation pathway in soil-derived *Burkholderia* (10, 18). (*B*) An adult male of *R. pedestris*. (*C*) A dissected alimentary tract of *R. pedestris*. An arrow indicates midgut fourth section as symbiotic organ. (*D*) An enlarged image of the symbiotic organ. (*E*) Localization of *Burkholderia* in the midgut crypts. Arrowheads indicate midgut crypts in *D* and *E*. (*F*) Halo formation by fenitrothion-degrading *Burkholderia* strains on a fenitrothion-containing agar plate. (*G*) Phylogenetic relationship of symbiotic *Burkholderia* strains from *R. pedestris* (orange) and soil-derived *Burkholderia* isolates (blue). Stars indicate fenitrothion-degrading strains. A maximum likelihood phylogeny inferred from 1,228 aligned nucleotide sites of 16S rRNA gene sequences is shown with bootstrap values. Closely related pairs of fenitrothion-degrading and nondegrading *Burkholderia* strains used in this study are highlighted by colored arrows: SFA1 and RPE67 in green, KM-A and RPE301 in red, and KM-G and RPE239 in blue.

were not different from those of the corresponding symbiotic *Burkholderia* strains (Fig. S2 *B–G*). Competitive infection assays revealed different levels of infectivity between the fenitrothion-degrading *Burkholderia* strains: SFA1 was as infective as the corresponding symbiotic strain RPE67, whereas KM-A and KM-G were much less infective than the corresponding symbiotic strains RPE301 and RPE239, respectively (Fig. 2*G*). These results indicate that at least some of the fenitrothion-degrading *Burkholderia* strains can efficiently establish a specific and beneficial symbiotic association with *R. pedestris*.

Fenitrothion-Degrading *Burkholderia* Symbionts Confer Insecticide Resistance on *R. pedestris*. When third instar nymphs of *R. pedestris* were reared on soybean seeds that had been dipped in fenitrothion solution and air-dried, the insects infected with the nondegrading *Burkholderia* strains showed high mortality, whereas the insects of the same genetic background infected with the corresponding fenitrothion-degrading *Burkholderia* strains exhibited significantly higher survival (Fig. 3*A*). Similar results were obtained under a different genetic background of *R. pedestris* (Fig. 3*B*). The resistance was observed not only with oral administration but also with percutaneous application of the insecticide (Fig. 3 *C* and *D*), which was rather unexpected on account of the midgut localization of the *Burkholderia* symbiont (Fig. 2 *A–F*). How the gut symbiont is involved in the in vivo detoxification processes should be clarified in future studies. The median lethal concentrations (LC_{50}) evaluated 24 h after fenitrothion treatment were 0.70 mM (95% confidence limit; 0.57–0.90 mM) for the insects infected with the fenitrothion-degrading strain SFA1 and 0.23 mM (0.19–0.27 mM) for the insects infected with the nondegrading strain RPE67. These results indicate that the fenitrothion-degrading *Burkholderia* symbionts confer an insecticide resistance on their host insects.

For fenitrothion-degrading bacteria, cross-acclimation against other organophosphorus insecticides has been reported (10, 18). We found that the fenitrothion-degrading *Burkholderia* strains SFA1, KM-A, and KM-G can degrade diazinon, *O*-ethyl *O*-(4-nitrophenyl) phenylphosphonothioate (EPN), and isoxathion to some extent (Table S3), suggesting a potentially broader impact of those symbionts on the insecticide resistance of their host insects.

Prevalence of Fenitrothion-Degrading Symbionts in Natural Populations of Stinkbugs. In our extensive field survey, all 846 individuals of *R. pedestris* from 13 localities exhibited no fenitrothion-degrading activity when their dissected symbiotic organ was subjected to the degradation assay (Table S4). Furthermore, we examined field populations of the rice bug *Leptocorisa chinensis*, which is known as

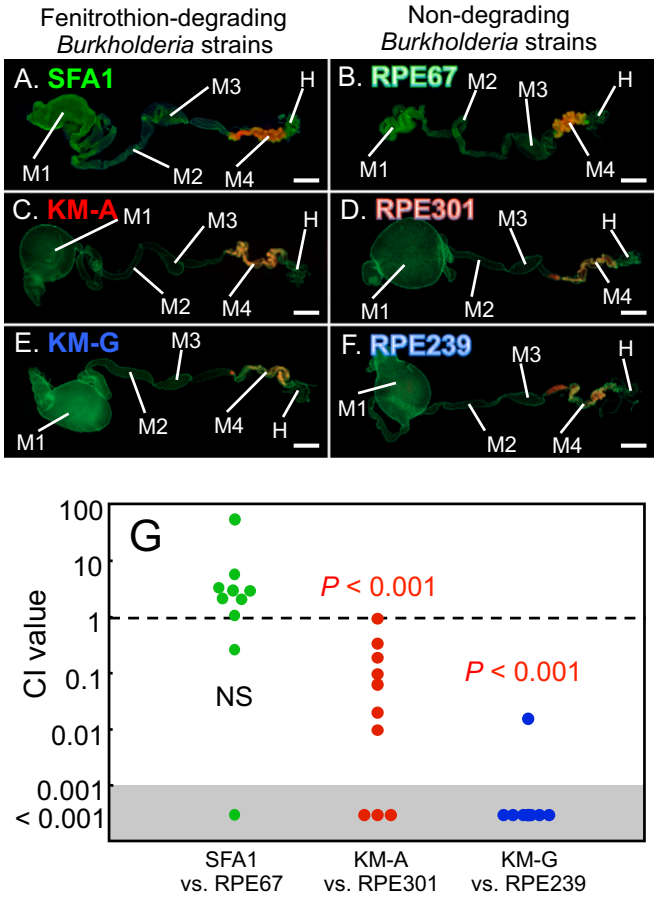

Fig. 2. Infectivity of fenitrothion-degrading *Burkholderia* strains to *R. pedestris*. (*A*–*F*) Fluorescent in situ hybridization of alimentary tract dissected from third instar nymphs that had been inoculated with soil-derived fenitrothion-degrading *Burkholderia* strains (*A*, SFA1; *C*, KM-A; *E*, KM-G) and nondegrading symbiotic *Burkholderia* strains (*B*, RPE67; *D*, RPE301; *F*, RPE239). Red and green signals indicate *Burkholderia* 16S rRNA and host insect nuclear DNA, respectively. M1, midgut first section; M2, midgut second section; M3, midgut third section; M4, midgut fourth section with crypts; H, hindgut. (Scale bars: 1 mm.) (*G*) Infection competitiveness of the fenitrothion-degrading *Burkholderia* strains against closely related, nondegrading *Burkholderia* strains: SFA vs. RPE67; KM-A vs. RPE301; and KM-G vs. RPE239. Each dot represents a competitive index (CI) value from an insect. The CI values are obtained by (output fenitrothion-degrading colony count)/(output nondegrading colony count) and statistically evaluated by the one sample *t* test. NS, not significant.

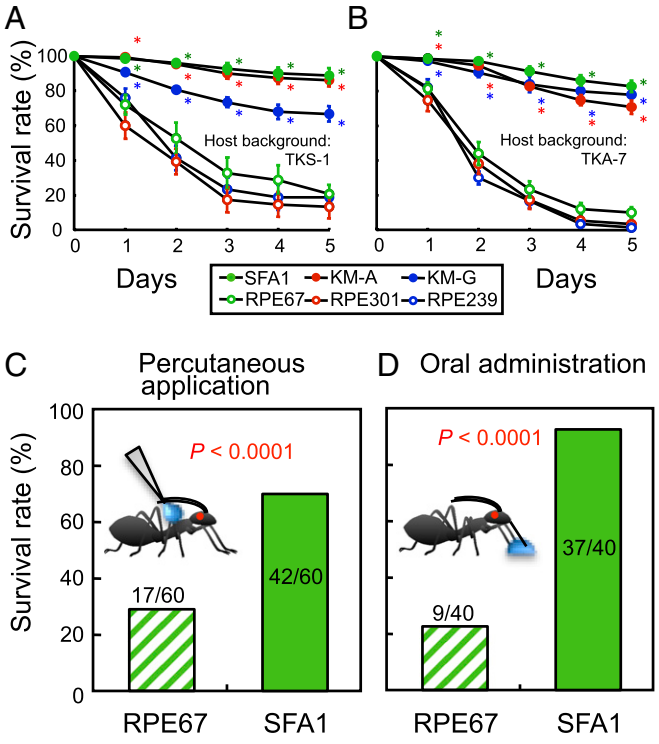

Fig. 3. Insecticide resistance of *R. pedestris* infected with fenitrothion-degrading *Burkholderia* strains. (*A* and *B*) Survival of third instar nymphs of *R. pedestris* infected with the fenitrothion-degrading and nondegrading *Burkholderia* strains when reared on fenitrothion-coated soybean seeds. Results under the host genetic background TKS-1 (*A*) and TKA-7 (*B*) are shown. Mean and SE of 10 replicates are indicated at each data point. Each asterisk indicates that survival rate of the insects infected with the fenitrothion-degrading *Burkholderia* strain is significantly higher than survival rate of the insects infected with the allied nondegrading strain (likelihood ratio test; $P < 0.01$). (*C* and *D*) Resistance of *Burkholderia*-infected *R. pedestris* to percutaneous application (*C*) and oral administration (*D*) of fenitrothion. Third instar nymphs, which were infected either with the fenitrothion-degrading *Burkholderia* strain (SFA1) or with the nondegrading *Burkholderia* strain (RPE67), were administrated with 30 pmol of fenitrothion, and their survival was inspected 24 h later. On each of the columns is shown number of surviving insects/total number of treated insects. Statistically significant differences in the survival rates are shown (Fisher's exact probability test).

a pest of rice (11) and also associated with the *Burkholderia* gut symbiont (12, 17). All 542 individuals from seven localities exhibited no significant fenitrithion-degrading activity in the symbiotic organ (Table S4). A previous study reported that, without enrichment by intensive insecticide applications, densities of fenitrothion-degrading bacteria in crop fields are usually <100 colony formation units (CFU) per g of soil (8). In Japan, farmers generally restrain heavy dose and frequent spraying of organophosphorus insecticides, which may be the reason for our failure to detect fenitrothion-degrading symbionts in field populations of the pest stinkbugs.

Fenitrothion Applications to Field Soils Enhance Infection with Fenitrothion-Degrading *Burkholderia* Symbionts in *R. pedestris*. We collected a soil sample from a soybean field at Tsukuba (Tsukuba-S), and prepared six pots containing the field soil. Each of three pots was treated with fenitrothion-containing water every week, whereas each of the other three pots was treated with distilled water. After 1 month, the control soils contained 10^4 to 10^6 bacterial CFU per gram, of which no colony exhibited fenitrothion-degrading activity. By contrast, the fenitrothion-treated soils contained 10^7 to 10^8 bacterial CFU per g, of which >80% of the colonies exhibited fenitrothion-degrading activities (Fig. 4*A*). In each of the control pots and the fenitrothion-treated pots, three soybean seedlings were planted, wherein 15 hatchlings of *R. pedestris* were reared to adulthood. Strikingly, most of adult insects from the treated pots exhibited significant fenitrothion-degrading activities in the symbiotic organ, whereas no adult insects from the control pots did so (Fig. 4*B*). Similar results were obtained with a soil sample from an adzuki bean field at Tsukuba (Tsukuba-A) (Fig. S3 *A* and *C*) and also with a soil sample from a soybean field at Kumamoto (Kumamoto-S) (Fig. S3 *B* and *D*).

We isolated the fenitrothion-degrading bacteria from the midgut of the adult insects, measured their fenitrothion-degrading activities (Table S5), and sequenced their 16S rRNA gene. Molecular phylogenetic analyses demonstrated that (*i*) all of the fenitrothion-degrading bacterial strains belong to the genus *Burkholderia*, (*ii*) the majority of them are placed in the *Burkholderia* symbiont clade, whereas some of them constitute a distinct sister clade, and (*iii*) different combinations of genetically distinct fenitrothion-degrading *Burkholderia* strains are enriched in the fenitrothion-treated Tsukuba-S, Tsukuba-A, and Kumamoto-S soils, respectively

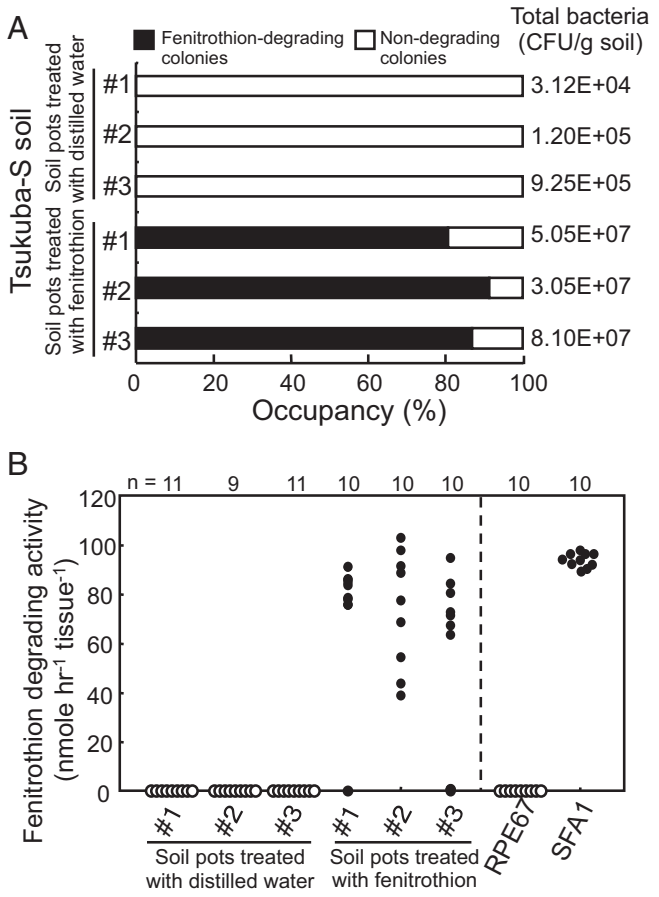

Fig. 4. Enrichment of fenitrothion-degrading *Burkholderia* strains in field-collected soils after experimental fenitrothion treatment. (*A*) Enrichment of fenitrothion-degrading bacteria in soil samples from a soybean field (Tsukuba-S) after weekly fenitrothion treatments for a month. Three soil pots were treated with fenitrothion-containing water (50 μg/g of soil each pot each time), whereas the other three control soil pots were treated with distilled water. (*B*) Fenitrothion-degrading activities in the symbiotic organ dissected from adult insects of *R. pedestris* that were reared in the fenitrothion-treated pots and the control pots, respectively. Each dot indicates activity value from an insect. Infection rates with fenitrothion-degrading bacteria are significantly different between the treatments (Fisher's exact probability test; $P < 0.0001$). Activity data from insects artificially infected with the fenitrothion-degrading *Burkholderia* strain SFA1 and the non-degrading symbiotic *Burkholderia* strain RPE67 are also shown.

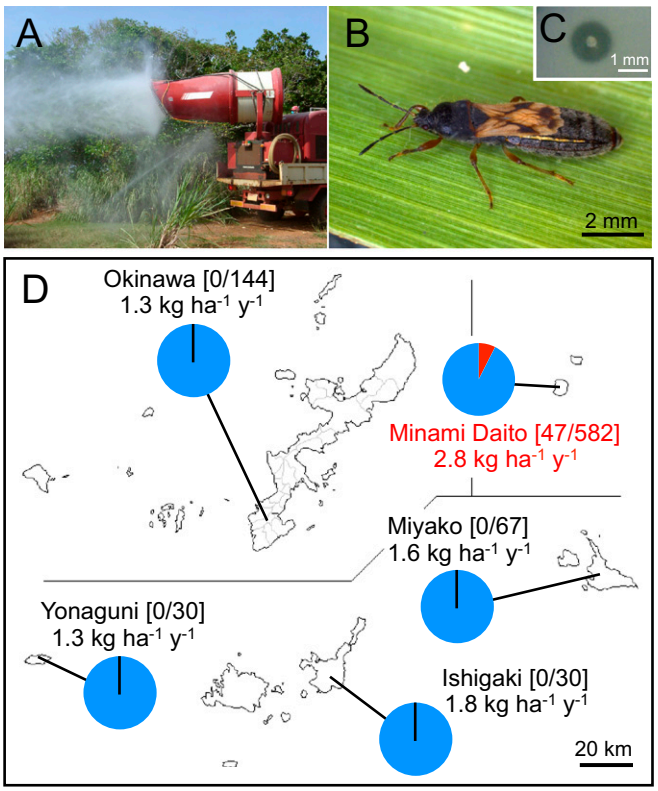

Fig. 5. Discovery of fenitrothin-degrading *Burkholderia* infection in *C. saccharivorus* at Minami-Daito Island, Japan. (*A*) Fenitrothion spraying in a sugarcane field at Minami-Daito Island. (*B*) An adult female of *C. saccharivorus*. (*C*) Degradation of fenitrothion by a *Burkholderia* strain (MDT2) isolated from *C. saccharivorus*. (*D*) Infection frequencies with fenitrothion-degrading (red) and nondegrading (blue) *Burkholderia* symbionts in *C. saccharivorus* populations in subtropical islands of Japan. The number of infected insects per number of all insects examined is shown in brackets. Mean annual use of fenitrothion in each of the islands is also shown [the data was from the Japan Agricultural Cooperatives, Okinawa office (JA Okinawa), and the Annual Report of Sugarcane in Okinawa Prefecture (Okinawa Prefectural Government)].

(Fig. S4). These results indicate that fenitrothion-degrading *Burkholderia* strains are present in field soils but at very low densities, constant fenitrothion applications to the soils drastically enrich them, and under such a condition *R. pedestris* readily acquires them in the symbiotic organ.

Natural Infection of Fenitrothion-Degrading Symbiont in a Pest Stinkbug Population. In Japan, we looked for agricultural fields where fenitrothion has been intensively used. Minami-Daito Island is a small, subtropical, and remote island, wherein flat areas are mostly used for sugarcane cultivation and have been administrated with fenitrothion at an annual dose of 2.8 kg per hectare for at least 5 y (Fig. 5 *A* and *D*). The oriental chinch bug *Cavelerius saccharivorus* (Fig. 5*B*) is among the major pests of sugarcane in subtropical Asia (11) and harbors *Burkholderia* symbiont in the midgut (17). Among adult insects of *C. saccharivorus* collected at sugarcane fields in Minami-Daito, 8% (47/582) exhibited remarkable fenitrothion-degrading activities in the posterior midgut (Fig. 5 *C* and *D* and Table S4). The *Burkholderia* isolates from the symbiotic organ of these insects certainly showed significant fenitrothion-degrading activities (Table S6), were genetically allied to the fenitrothion-degrading *Burkholderia* from *R. pedestris* (Fig. S4), and conferred a significant resistance to fenitrothion on experimentally infected *R. pedestris* (Fig. S5). In other Japanese subtropical islands, we have not yet detected fenitrothion-degrading *Burkholderia* from *C. saccharivorus* (Fig. 5*D* and Table S4).

Conclusion and Perspective. We discovered a symbiont-mediated mechanism of insecticide resistance in pest stinkbugs. Previous studies showed that symbiotic bacteria can be involved in insect resistance to biological insecticides like *Bacillus thuringiensis* and parasitoid wasps (19, 20). Our finding extends such phenomena to chemical insecticides that are much more widely used and important for world's agriculture and public health. The symbiont-mediated insecticide resistance is conceptually unprecedented in the following respects. First, insecticide applications enrich the insecticide-degrading bacteria in the agroecosystem, which predisposes the development of insecticide resistance even in the absence of pest insects. Second, upon emergence and/or migration of pest insects in the enriched agroecosystem, they can immediately acquire the symbiont and become resistant to the insecticide, establishing the insecticide resistance very quickly (potentially within a single insect generation), which is in contrast to the slower mutation-selection process acting on the pest insect genomes. Third, the symbiont infection confers not only insecticide resistance but

also fitness benefits on the host insects, which may facilitate the spread of the resistant trait in the pest insect populations. Fourth, the association with the host insects may contribute to proliferation and dispersal of the insecticide-degrading bacteria, because the host insects amplify the bacteria in the symbiotic organ and actively fly and migrate. Finally, because of the infectious nature of the causative agent, the resistant trait may potentially move around horizontally between different pest insects and other organisms. Whether these processes are operating in the real agroecosystem is of not only scientific but also practical interest, which deserves future studies on *C. saccharivorus* populations in sugarcane fields at Minami Daito and other subtropical islands.

Materials and Methods

Insects and Bacterial Strains. Information on field-collected insects of *R. pedestris*, *L. chinensis,* and *C. saccharivorus* is summarized in Table S4. For fenitrothion treatment and rearing experiments, isofemale lines (TKS1 and TKA7) of *R. pedestris* were established from insects collected at Tsukuba, Ibaraki, Japan. The insect cultures were maintained on soybean seeds and distilled water containing 0.05% ascorbic acid (DWA) at 25 °C under a long-day regime (16 h light, 8 h dark). The *Burkholderia* strains used in this study are three fenitrothion-degrading isolates from field soils and three non-degrading isolates from *R. pedestris* (Tables S1–S3), 35 fenitrothion-degrading isolates from *R. pedestris* reared with fenitrothion-enriched soils (Table S5), and three fenitrothion-degrading isolates and three non-degrading isolates from *C. saccharivorus* (Table S6). These bacterial strains were stored as frozen stocks at −80 °C and cultured at 25 °C on YG agar plates (5 g of yeast extract, 4 g of glucose, 1 g of NaCl, and 15 g of Bacto agar per liter of distilled water) as described (13). The *Burkholdeia* strains were orally administered to second instar nymphs of *R. pedestris* as described (16).

Detection and Quantification of Fenitrothion-Hydrolyzing Activities in Bacterial Isolates. Qualitative detection of fenitrothion-hydrolyzing activities was based on halo formation around bacterial colonies on YG agar plates containing 0.08% fenitrothion emulsion. Quantitative measurement of fenitrothion-hydrolyzing activities was performed by HPLC as described (8, 10). For the HPLC assay, $\approx 7.0 \times 10^7$ *Burkholderia* cells were incubated in 1.0 mL of 20 mM sodium-potassium phosphate buffer (pH 7.0) containing 1.0 mM fenitrothion. After 20 min of incubation at 30 °C, the reaction was stopped by adding an equal volume of methanol, and then fenitrothion and its metabolite, 3-methyl-4-nitrophenol, were analyzed by a HPLC system (consisted of 600E pump and 2487 dual absorbance detector; Waters). Retention times and peak areas of the HPLC profiles were compared with those of known standards. Hydrolysis of other organophosphrus insecticides, including diazinon, EPN, and isoxathion, was determined and quantified by the HPLC assay under the same reaction condition.

Fenitrothion Treatment of Insects. Soybean seeds were dipped in 0.2 mM fenitrothion for 5 s and dried at room temperature. In each clean plastic container, 15 newly molted third instar nymphs of *R. pedestris* were reared on five fenitrothion-treated soybean seeds and DWA at 25 °C under the long-day regime. For quantitative percutaneous application, 1 μL of acetone containing 30 μM fenitrothion was applied onto the dorsal thoracic plate of each newly molted third instar nymph. For quantitative oral application, 1 μL of DWA containing 30 μM fenitrothion was fed to each newly molted third instar nymph.

Survey of Fenitrothion-Degrading Symbionts in Field Insect Populations. Field-collected insects were surface-sterilized with 70% ethanol and dissected in a PBS. The dissected symbiotic organs containing the symbiotic bacteria were individually homogenized in 100 μL of fenitrothion buffer (0.2 mM fenitrothion, 50 mM Tris·HCl at pH 8.0, and 0.1% Triton X-100), incubated at 25 °C for 1 h without shaking, and subjected to measurement of the optical density of 3-methyl-4-nitrophenol at 405 nm (8, 10) on 96-well plates by using a plate reader (DIGI Scan; ASYS Hitech). *Burkholderia* strains were isolated from the positive wells and subjected to the HPLC assay to confirm their fenitrothion-hydrolyzing activities.

Enrichment of Fenitrothion-Degrading Bacteria in Field-Collected Soils. We prepared 6–10 pots containing 300 g of soil from each of the soil samples. They were divided into two groups: one group was treated with 100 mL of fenitrothion-containing water every week at a dose of 50 μg/g of soil, whereas the other group was treated with 100 mL of distilled water every week. After a month, 0.1 g of soil from each of the pots was suspended with distilled water and plated onto minimal agar plates containing 0.08% fenitrothion emulsion (8). Colonies with and without halo were counted on the plates after 5 d of incubation at 25 °C.

Insect Rearing on Fenitrothion-Enriched Soil. In each of the fenitrothion-treated and control soil pots, three soybean seedlings were planted, five soybean seeds were provided, and 15 hatchlings of *R. pedestris* were introduced. During the rearing period for 2 wk, the soybean plants were watered from the bottom of the pots every 2 d, and the soybean seeds were renewed every 4 d. After adult emergence, the symbiotic organ was dissected from each insect, homogenized in 100 μL of fenitrothion buffer, incubated at 25 °C for 15 min without shaking, and subjected to measurement of the optical density of 3-methyl-4-nitrophenol at 400 nm (8, 10) by using a spectrophotometer (NanoDrop 1000; Thermo Fisher Scientific). *Burkholderia* strains were isolated from the positive samples and subjected to the HPLC assay to confirm their fenitrothion-hydrolyzing activities.

Supporting Materials and Methods. Further methodological details are described in *SI Materials and Methods*.

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