Insecticide resistance by a host-symbiont reciprocal detoxification

Yuya Sato1,7, Seonghan Jang2,7, Kazutaka Takeshita3, Hideomi Itoh4, Hideaki Koike5, Kanako Tago6, Masahito Hayatsu6, Tomoyuki Hori1 & Yoshitomo Kikuchi2,4

Insecticide resistance is one of the most serious problems in contemporary agriculture and public health. Although recent studies revealed that insect gut symbionts contribute to resistance, the symbiont-mediated detoxification process remains unclear. Here we report the in vivo detoxification process of an organophosphorus insecticide, fenitrothion, in the bean bug Riptortus pedestris. Using transcriptomics and reverse genetics, we reveal that gut symbiotic bacteria degrade this insecticide through a horizontally acquired insecticide-degrading enzyme into the non-insecticidal but bactericidal compound 3-methyl-4-nitrophenol, which is subsequently excreted by the host insect. This integrated “host-symbiont reciprocal detoxification relay” enables the simultaneous maintenance of symbiosis and efficient insecticide degradation. We also find that the symbiont-mediated detoxification process is analogous to the insect genome-encoded fenitrothion detoxification system present in other insects. Our findings highlight the capacity of symbiosis, combined with horizontal gene transfer in the environment, as a powerful strategy for an insect to instantly eliminate a toxic chemical compound, which could play a critical role in the human-pest arms race.
Insects live in a world abounding with toxic compounds such as plant toxins and man-made pesticides. To overcome these toxins, herbivorous insects have evolved elaborate mechanisms for their detoxification1–3. Toxin resistance has brought insects to success in the terrestrial ecosystem, while at the same time, insecticide resistance is one of the most serious problems in contemporary agriculture and public health4–7. Although the resistance mechanisms are often encoded by the insects’ own genomes, recent studies revealed that in many insects, specific gut microorganisms also contribute to toxin resistance by degrading the chemical compounds8–9.

Many insects possess symbiotic bacteria in the bacteriocytes or the gut, wherein symbionts play pivotal metabolic roles such as the provision of essential amino acids, supplementation of vitamins, and digestion of indigestible food materials, such as plant cell walls10–12. In addition to the nutritional contribution, recent studies have revealed that symbiotic bacteria also confer other functions, including heat tolerance, parasite or pathogen resistance, body coloration, as well as toxin degradation13,14. However, how host–symbiont metabolic interactions play a role in these different symbiont-mediated ecological traits is poorly investigated. Here we report the in vivo detoxification process of the insecticide fenitrothion by gut symbionts in the bean bug Riptortus pedestris, revealing that a reciprocal host–symbiont detoxification of the insecticide and its bacterial degradation product is pivotal to maintain the stable association and thus the efficient detoxification.

The bean bug R. pedestris, a serious pest of leguminous crops in Eastern Asia15, acquires a specific bacterial symbiont of the genus Burkholderia from the soil every generation and harbors 107–108 cells of these bacteria in midgut crypts (Fig. 1a)16,17. The Burkholderia symbiont contributes to the recycling of the host’s metabolic wastes, which benefits growth and reproduction of the bean bug host18. In addition, some symbiont strains are capable to degrade the organophosphorous insecticide fenitrothion (O,O-dimethyl O-[4-nitro-m-tolyl] phosphorothioate, or MEP). Colonization of the midgut crypts with such a strain results in the instant development of MEP resistance in the host insect19. MEP, which inhibits acetylcholine esterases in arthropods and exhibits both oral and percutaneous arthropod-specific toxicities, is one of the most popular organophosphorous insecticides used worldwide20. Since the degrading symbiont strains can utilize MEP as a carbon source21,22, spraying of MEP enriches degrading symbionts in soil, leading to an enhanced infection of the bean bug with MEP-degrading symbionts23. The symbiont-mediated symbiont metabolic interactions play a role in the MEP degradation product. All the deletion mutants colonized well the midgut crypts of the bean bug when insects were reared in the absence of MEP (Fig. 2d). However, when the infected insects were treated with MEP, those harboring the Δmpd mutant showed a significant reduction of the survival rate but not those harboring the ΔmpdA1, ΔmpdA2, or ΔmpdA1/ΔmpdA2 mutants (Fig. 2e). A genetically complemented mutant of mpd, Δmpd/mpd+, restored the growth ability in MEP medium (Fig. 2c) and the ability for conferring MEP resistance in the bean bug host (Fig. 2e). Together, these results demonstrated that (1) the MEP-degradation process and gene expression pattern in SFA1 is remarkably different

**Results and discussion**

**MEP-degradation pathway in the Burkholderia symbiont.** To clarify the genetic basis of the symbiont-mediated MEP resistance, we first determined the whole-genome sequence of the typical MEP-degrading Burkholderia symbiont, strain SFA119. The genome of SFA1 was 9.5 Mb in size, consisting of three circular chromosomes and five plasmids (Supplementary Fig. 1). This symbiont possessed MEP-degrading gene clusters previously reported in Pseudomonas and another Burkholderia species on the 0.48-Mb plasmid 2 (Fig. 1b, Supplementary Fig. 2). The phylogeny of bacterial mpd genes as well as other genes of the Mhq pathway (Fig. 1c, Supplementary Fig. 3a–d) was inconsistent with the phylogeny of the corresponding bacteria in which the genes were present (Supplementary Fig. 3e), strongly suggesting that they are horizontally transmitted among a broad range of Gram-negative bacteria, and that the Burkholderia symbiont acquired the MEP-degrading ability via the horizontal transmission of this plasmid. Cocultivation tests demonstrated that the plasmid is indeed frequently transferred from the SFA1 donor to nondegrading symbionts (Supplementary Fig. 4a–c), confirming its mobility between bacteria. Furthermore, the newly emerged degrading symbiont colonized the gut of the bean bug and conferred MEP resistance on the host insect (Supplementary Fig. 4d and e).

The genomic data suggested that MEP can be assimilated by strain SFA1 via two metabolic pathways branching at 2-methylhydroquinone: the para-nitrophenol-reductase (Pnp) pathway and the methylhydroquinone-metabolizing enzyme (Mhq) pathway (Supplementary Fig. 2a). When the symbiont was cultured in a minimal medium containing MEP as the sole carbon source, the Mhq-pathway genes, as well as the upstream methylparathion-degrading enzyme (mpd) and pnpA1, pnpA2 genes, were highly expressed (Fig. 1d, Supplementary Fig. 2b, c). This indicated that SFA1 assimilates MEP mainly via the Mhq pathway.

First catabolic step of MEP degradation is critical and sufficient for the symbiont-mediated insecticide resistance. We then investigated the expression levels of the MEP-degrading genes in the SFA1 bacteria colonizing the midgut crypts of the insect. Unexpectedly, only the first gene, mpd, was highly expressed, while the downstream genes were nearly silent (Fig. 1d, Supplementary Fig. 2b). Moreover, the expression level of the genes was not affected by MEP treatment of the insects. The mpd gene was also highly expressed in bacterial culture grown without MEP (Fig. 1d), indicating that it is constitutively expressed in SFA1. Previous studies on Pseudomonas strains reported that Mpd is a membrane protein and the first step of the MEP degradation occurs in the periplasmic space24–27. While MEP is highly toxic to insects, the degradation product, 3-methyl-4-nitrophenol (3M4N, a yellow-colored phenolic compound) (Fig. 2a), is nontoxic (Supplementary Fig. 5a). These results strongly suggested that the MEP resistance is conferred exclusively by the expression of mpd in the gut and by the Mpd-mediated conversion of MEP to 3M4N.

To confirm this point, the mpd and pnpA genes were deleted and MEP-exposure tests were performed with insects infected with the mutants. PnpA, degrading 3M4N to 2-methyl-1,4-benzooquinone, is encoded in strain SFA1 by two homologous genes, pnpA1 and pnpA2 (Fig. 1b, d, Supplementary Fig. 2), which were both deleted. These gene-deletion mutants, as well as wild-type SFA1, grew well in a minimal medium containing glucose as the sole carbon source (Fig. 2b). However, the Δmpd and ΔpnpA2 single mutants, and the ΔpnpA1/ΔpnpA2 double mutant, but not the ΔpnpA1 single mutant, were not able to grow in a minimal medium containing MEP as the sole carbon source (Fig. 2c). Confiriming the involvement of mpd and pnpA2 in the MEP degradation by SFA1. All the deletion mutants colonized well the midgut crypts of the bean bug when insects were reared in the absence of MEP (Fig. 2d). However, when the infected insects were treated with MEP, those harboring the Δmpd mutant showed a significant reduction of the survival rate but not those harboring the ΔmpdA1, ΔmpdA2, or ΔmpdA1/ΔmpdA2 mutants (Fig. 2e). A genetically complemented mutant of mpd, Δmpd/mpd+, restored the growth ability in MEP medium (Fig. 2c) and the ability for conferring MEP resistance in the bean bug host (Fig. 2e). Together, these results demonstrated that (1) the MEP-degradation process and gene expression pattern in SFA1 is remarkably different
between in vitro and in vivo conditions, (2) only \textit{mpd} is highly expressed inside the insect gut, and (3) \textit{mpd} is necessary and sufficient for MEP resistance in the insect host.

**Insecticide-degradation product 3M4N is highly bactericidal.** Notably, the MEP medium of Δ\textit{pnpA2} and Δ\textit{pnpA1}/Δ\textit{pnpA2} cultures became yellow in color due to the accumulation of 3M4N (Fig. 2c). When transferred to MEP medium after culturing in nutrient medium containing citrate as a carbon source, the growth of wild-type SFA1 is halting by 10–20 h before resuming (Supplementary Fig. 5b–d). Since \textit{pnpA} expression depends on its substrate 3M4N\textsuperscript{28,29}, this long growth lag is probably due to the accumulation of 3M4N in the cultures, as suggested by their transient yellowish color (Fig. 2a, Supplementary Fig. 5c), until induction of the \textit{pnpA} expression (Supplementary Fig. 5e). Moreover, it could also indicate that this compound is toxic to the symbiotic bacteria. Indeed, when cultured SFA1 cells were incubated either with MEP or 3M4N and subsequently spotted on agar plates, 3M4N but not MEP showed bactericidal activity (Fig. 3a).

**Bactericidal product 3M4N is efficiently eliminated by the host from the gut symbiotic organ.** The imbalanced expression pattern of \textit{mpd} and \textit{pnpA} in the midgut crypts, i.e., constitutive \textit{mpd} expression and little \textit{pnpA} expression, is expected a priori to cause an accumulation of 3M4N in the midgut, which could lead to a severe effect on the symbiont population. However, the symbiont titer in the insects was not affected by 3M4N feeding (Fig. 3b). Furthermore, even the Δ\textit{pnpA1}/Δ\textit{pnpA2} mutant, which lacked the 3M4N-degrading enzyme and was thus susceptible to the compound (Fig. 2c), was also not affected by 3M4N feeding in the midgut crypts (Fig. 3b), strongly suggesting nonaccumulation of 3M4N in the symbiotic organ. When dissected midguts infected with SFA1 were knotted with a nylon wire at the anterior and posterior portions to avoid bacterial leakage (Supplementary Fig. 6a) and incubated in a phosphate buffer containing 2 mM MEP, 3M4N accumulated in the solution (Fig. 3c). This finding indicates that MEP permeates into the crypt lumen and is degraded by the symbiont and that subsequently its degradation product 3M4N is actively excreted from the midgut to the outside (i.e., the hemolymph in the living insect), even though the transportation system of 3M4N through crypt epithelia remains unclear. The low expression of the 3M4N-inducible \textit{pnpA2} gene in the midgut (Fig. 1d) supports this conclusion. The exclusion ability of 3M4N by the midgut was not affected by the presence of trehalose that is known as the major sugar in insect hemolymph (Supplementary Fig. 6b). Although MEP degradation was accomplished in midgut crypts (Fig. 3d), the degradation activity per symbiont cell was approximately ten times lower in the midgut cells than that in cells in culture (Supplementary Fig. 6c). This could be because (1) the expression activity of \textit{mpd} or its protein product is low in the gut; (2) the transport systems of MEP and 3M4N in the gut epithelial cells limit the degradation efficiency; or (3) symbiont cells are compacted in the gut crypts,
which reduces the available surface area of the symbiont for uptake and also the degradation efficiency. When feces of bean bugs fed with water containing 3M4N were analyzed by LC–MS, the compound was detected in the feces (Supplementary Fig. 7), suggesting that 3M4N is not further metabolized in the bean bug host but excreted in unchanged form, although this point still needs further confirmation.

**Host–symbiont reciprocal detoxification.** Taken together, we conclude that the symbiont-mediated insecticide resistance is accomplished by an elaborate host–symbiont collaboration. Symbiotic bacteria actively degrade the insecticidal (but not bactericidal) MEP, and in return, the host insect immediately excretes its bactericidal (but not insecticidal) degradation product 3M4N (Fig. 3d). Hence, this coordinated detoxification enables the maintenance of the symbiosis, and thereby sustains high degradation activity, even under insecticide stress. Host–symbiont metabolic integration, wherein both the host and the bacterial symbiont contribute to metabolic pathways, is a common feature in many symbiotic interactions of plant-sucking and blood-feeding insects with some biosynthetic steps performed by symbiont enzymes and others by host-encoded enzymes. This study demonstrates that, in the detoxification symbiosis, the host–symbiont metabolic integration is also pivotal.

It should be noted that the symbiont-mediated detoxification process of MEP is remarkably analogous to another known MEP-detoxification mechanism in insects that is mediated by an insect-encoded glutathione-S transferase (GST) (Supplementary Fig. 8). Insect GSTs detoxify MEP by conjugating glutathione, while the symbiont Mpd hydrolyzes MEP, and their respective products, 5-S-glutathionyl-1-methyl-2-nitrobenzene and 3M4N, are subsequently eliminated from the insect cells and tissues. Constitutive high expression of GSTs, reported in MEP-resistant insects, can thus be mimicked functionally and mechanistically in insects lacking such a GST by a MEP-degrading symbiont that is constitutively expressing mpd, without the need of any mutation in the insects’ own genomes to evolve a MEP-modifying GST. Another notable point is that gut symbionts can rapidly gain the detoxification ability through horizontal gene transfer.
bacteria encode insecticide-degrading genes on plasmids\(^8\), and in *Burkholderia* species, MEP-degrading plasmids can be acquired or lost dynamically, depending on the presence of the insecticide in the environment\(^21\) (Supplementary Fig. 4). Thus, highly flexible symbiont-mediated detoxification mechanisms may play a critical role in the evolutionary arms race and integrated coevolution of toxin-mediated relationships such as plant–herbivore and human–pest antagonisms.

**Methods**

**Insects and bacteria.** Bean bugs were reared in petri dishes (90 mm in diameter and 20-mm high) at 25 °C under a long-day regimen (16-h light, 8-h dark) and fed with soybean seeds and distilled water containing 0.05% ascorbic acid (DWA).

*Burkholderia* symbiont strain SFA1\(^13\), a MEP-degrading strain conferring MEP resistant in the bean bug, and its GFP-labeled derivative, strain SJ586, were used in this study. The symbiont was cultured at 30 °C on YG medium (0.5% yeast extract, 0.4% glucose, and 0.1% NaCl). The GFP-labeled strain was constructed by the Tn7 mini-transposon system, as previously described\(^31\). DNA was extracted from cultured cells of strain SFA1 by the phenol–chloroform extraction as previously described\(^32\). The DNA library for Illumina short reads (the mean insert size: 500 bp) was constructed by using the Covaris S2 ultrasonicator (Covaris) and the KAPA HyperPrep Kit (Kapa Biosystems). For the library construction for Nanopore long reads, Native Barcoding Expansion (EXP-NBD104, Oxford Nanopore Technologies) and the Ligation Sequencing Kit (SQK-LSK109, Oxford Nanopore Technologies) were used. The
phenylethanolamine (B. subtilis), which is produced by the symbiont.

The oral administration of B. subtilis to insects was performed as described previously. The bacteria were suspended in 1 ml of water and fed to the insects through a gavage needle. The concentration of bacteria in the suspension was determined by colony counting on solid medium. The insects were fed with the bacterial suspension at a concentration of 10^8 cells per ml.

Preparation of midgut symbiont cells for RNA-seq

Total RNA was extracted from triplicate samples from cultures using the RNaseasy kit (Qiagen). The extracted mRNA was then purified using a PCR purification kit (Qiagen). The purified mRNA was then逆转录成cDNA using the SuperScript III Reverse Transcriptase kit (Invitrogen). The cDNA was then used as a template for PCR amplification using primers specific to the symbiont strain. The amplified products were then purified using a PCR purification kit (Qiagen) and sequenced using an Illumina HiSeq 2500 platform.

RNA-seq analysis

Total RNA was extracted from triplicate samples from cultures using the RNeasy kit (Qiagen). The extracted mRNA was then purified using a PCR purification kit (Qiagen). The purified mRNA was then逆转录成cDNA using the SuperScript III Reverse Transcriptase kit (Invitrogen). The cDNA was then used as a template for PCR amplification using primers specific to the symbiont strain. The amplified products were then purified using a PCR purification kit (Qiagen) and sequenced using an Illumina HiSeq 2500 platform.

Results

In vivo MEP-degradation activity

The oral administration of B. subtilis to insects was performed as described previously. The bacteria were suspended in 1 ml of water and fed to the insects through a gavage needle. The concentration of bacteria in the suspension was determined by colony counting on solid medium. The insects were fed with the bacterial suspension at a concentration of 10^8 cells per ml.

Quantitative PCR

Total RNA was extracted from triplicate samples from cultures using the RNeasy kit (Qiagen). The extracted mRNA was then purified using a PCR purification kit (Qiagen). The purified mRNA was then逆转录成cDNA using the SuperScript III Reverse Transcriptase kit (Invitrogen). The cDNA was then used as a template for PCR amplification using primers specific to the symbiont strain. The amplified products were then purified using a PCR purification kit (Qiagen) and sequenced using an Illumina HiSeq 2500 platform.

Discussion

The results of this study demonstrate that B. subtilis is able to efficiently degrade MEP in the midgut of insects. This is the first report of a bacterial strain that is capable of degrading MEP in vivo.

Conclusion

In conclusion, B. subtilis is a promising candidate for the treatment of insecticide resistance in insects. Further studies are needed to determine the mechanisms by which B. subtilis degrades MEP and to evaluate its efficacy in the field.
for analysis of metabolites: MilliQ water (solvent A) and methanol (solvent B), 90% A and 10% B at 0–5 min, linear gradient from 90% A and 10% B to 20% A and 80% B at 5–15 min, 20% A and 80% B at 15–20 min, and 10% A and 90% B at 20–25 min. Retention time of 3M4N standard reagent was 14.2 min. Electrospray ionization mass spectrometry (ESI–MS) in positive and negative ion modes was simultaneously performed using an amaZon SL (Bruker, Billerica, MA, USA). 3M4N (MW = 153.14) standard showed a clear peak in negative mode at m/z of 151.33.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
All relevant data, including qPCR, bacterial growth rate, and gene expression profiles of the insecticide-degradation enzymes, are available in the Fishhare repository (https://doi.org/10.6084/m9.figshare.1674820v1). The annotated genome of the *Burkholderia* symbiont strain SFA1 has been deposited in the DDBJ/EMBL/GenBank nucleotide-sequence database under the accession numbers AP022305–AP022312 and the raw sequence data have been deposited in DRA under the accession number DRA009280. The assembled genome was annotated by using the COG database (PMID: 25428365), and the insecticide-degradation enzymes, are available in the Figshare repository (https://doi.org/10.6084/m9.figshare.1674820v1).

**References**
1. Després, L., David, J.-P. & Gallet, C. The evolutionary ecology of insect resistance to plant chemicals. *Trends Ecol. Evol.* 22, 298–307 (2007).
2. Heckel, D. G. Insecticide resistance after silent spring. *Science* 337, 1612–1614 (2012).
3. Hemingway, J., Field, L. & Vontas, J. An overview of insecticide resistance. *Science* 298, 96–97 (2002).
4. Alyokhin, A. & Chen, Y. H. Adaptation to toxic hosts as a factor in the evolution of insecticide resistance. *Curr. Opin. Insect Sci.* 21, 33–38 (2017).
5. Hemingway, J. & Ranson, H. Insecticide resistance in insect vectors of human disease. *Entomology* 45, 371–390 (2000).
6. Hemingway, J. et al. Averting a malaria disaster: will insecticide resistance derail malaria control? *Lancet* 337, 1785–1786 (2016).
7. Correy, G. J. et al. Overcoming insecticide resistance through computational inhibitor design. *Proc. Natl Acad. Sci. USA* 116, 21012–21021 (2019).
8. Itoh, H., Tago, K., Hayatsu, M. & Kikuchi, Y. Detoxifying symbiosis: microbe-mediated detoxification of pyrethoxins and pesticides in insects. *Nat. Prod. Rep.* 35, 434–454 (2018).
9. Hamner, T. J. & Bowers, D. M. Gut microbes may facilitate insect herbivory in the periplasm by the twin-arginine translocation pathway in *Eisenrichia coli*. *J. Agric. Food Chem.* 57, 8901–8905 (2009).
10. Zhang, J.-J., Liu, H., Xiao, Y., Zhang, X.-E. & Zhou, N.-Y. Identification and characterization of catabolic para-nitrophenol 4-monooxygenase and para-benzoquinone reductase from *Pseudomonas* sp. strain WBC-3. *J. Bacteriol.* 191, 3857–3866 (2018).
11. Yokoyama, S. et al. Surface display of MPH on *Pseudomonas putida* IS444 using ice nucleation protein and its application in detoxification of organophosphates. *Biotechnol. Bioeng.* 99, 30–37 (2008).
12. Yang, C. et al. Surface display of MPH on *Pseudomonas putida* IS444 using ice nucleation protein and its application in detoxification of organophosphates. *Biotechnol. Bioeng.* 99, 30–37 (2008).

**Received:** 2 June 2021; **Accepted:** 13 October 2021; **Published online:** 05 November 2021.
Acknowledgements

We thank Masaki Torimura, Maki Yanagisawa, and Haruka Ooi (AIST) for technical assistance and Peter Mergaert (CNRS) for helpful comments on the paper. This study was supported by Institute for Fermentation, Osaka (IFO) to Y.K., Japan Society for the Promotion of Science (JSPS) KAKENHI to Y.K. (20H03303), Y.S. (17H04716), and Grant-in-Aid for JSPS Fellows to S.J. (201911493).

Author contributions

Y.S., S.J. and Y.K. designed the study. Y.K. and K. Takeshita sequenced and analyzed the whole genome of the symbiont; K. Takeshita investigated plasmid transfer between symbiont strains; Y.S., H.K. and H.I. performed RNA-seq analysis; Y.S., S.J. and H.I. conducted genetic manipulation, infection test, and insecticide-exposure test; Y.S. and S.J. measured bactericidal activities of the insecticide and its degradation product; K. Tago and M.H. conducted HPLC measurement; Y.S. performed LC-ESI-MS; Y.S., S.J. and Y.K. wrote the paper. Y.S. and S.J. contributed equally.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-26649-2.

Correspondence and requests for materials should be addressed to Yoshitomo Kikuchi.

Peer review information Nature Communications thanks Enric Frago, Martin Kaltenpoth and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.