



Infection dynamics of insecticide-degrading symbionts from soil to insects in response to insecticide spraying

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Abstract

Insecticide resistance is a serious concern in modern agriculture, and an understanding of the underlying evolutionary processes is pivotal to prevent the problem. The bean bug *Riptortus pedestris*, a notorious pest of leguminous crops, acquires a specific *Burkholderia* symbiont from the environment every generation, and harbors the symbiont in the midgut crypts. The symbiont's natural role is to promote insect development but the insect host can also obtain resistance against the insecticide fenitrothion (MEP) by acquiring MEP-degrading *Burkholderia* from the environment. To understand the developing process of the symbiont-mediated MEP resistance in response to the application of the insecticide, we investigated here in parallel the soil bacterial dynamics and the infected gut symbionts under different MEP-spraying conditions by culture-dependent and culture-independent analyses, in conjunction with stinkbug rearing experiments. We demonstrate that MEP application did not affect the total bacterial soil population but significantly decreased its diversity while it dramatically increased the proportion of MEP-degrading bacteria, mostly *Burkholderia*. Moreover, we found that the infection of stinkbug hosts with MEP-degrading *Burkholderia* is highly specific and efficient, and is established after only a few times of insecticide spraying at least in a field soil with spraying history, suggesting that insecticide resistance could evolve in a pest bug population more quickly than was thought before.

Introduction

Chemical insecticides are used worldwide for controlling agricultural and medical pests. Their use has greatly

contributed to the progress of modern agriculture and public health. On the other hand, use of insecticides has frequently led to many problems, including the development of insecticide resistance in pest populations. Mechanisms underpinning the insecticide resistance are diverse, including alteration of drug target sites, upregulation of degrading enzymes and enhancement of drug excretion [1–3], all of which have been generally attributable to mutational changes in the pests' own genomes. Recently, we revealed a novel type of insecticide resistance in the bean bug *Riptortus pedestris* and phylogenetically related species, wherein the stinkbugs become resistant against an organophosphorus insecticide, fenitrothion, by acquiring fenitrothion-degrading symbionts from environmental soil [4]. Since this first report, symbiont-mediated insecticide resistance has been reported or suggested in other insects, including lepidopteran pests [5–7], *Drosophila* [8], and other fruit flies [9], but how the symbiont-mediated insecticide resistance develops over time, after application of the insecticide, has been poorly investigated.

Riptortus pedestris, known as a notorious pest of leguminous crops [10], is associated with a gut bacterial

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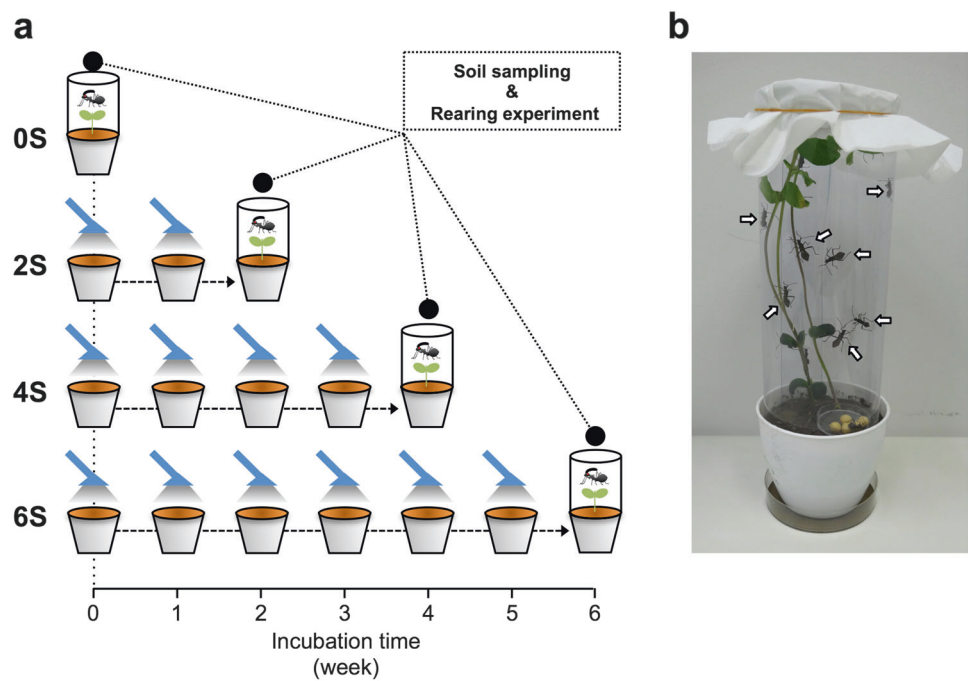
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Fig. 1 Experimental design to elucidate the infection dynamics of MEP-degrading bacteria from soil to insect. **a** Schema of the MEP-spraying treatment of soils and the insect rearing experiment. In addition to untreated soil (0S), field-collected soils were weekly treated with MEP either two times (2S), four times (4S), or six times (6S). One week after the final MEP-spraying, soil samples were collected from each of the MEP-treated pots, and the remaining soils were used for the insect rearing experiment. **b** An image of a soybean pot for the insect rearing experiment. Arrows indicate fourth instar nymphs of *R. pedestris*. After adult emergence, insects were collected and subjected to culture-dependent and culture-independent analyses, whose results were compared with those of soils



symbiont of the genus *Burkholderia* in a posterior region of the midgut [11–13]. In the specialized symbiotic organ, an adult insect harbors as many as 10^8 cells of the *Burkholderia* symbiont [14], enhancing growth and fecundity of the host insect [15], indicating the beneficial nature of the symbiotic association. In contrast to vertical symbiont transmission ubiquitously found in many insects [16], *R. pederis* acquires the symbiont from ambient soil every host generation [17]. Broad surveys have revealed that the *Burkholderia* symbiont is widely associated with the members of the stinkbug superfamilies Coreoidea and Lygaeoidea [11, 18–22]. The stinkbug-associated *Burkholderia* are genetically diverse but clustered in a specific clade, called “Stinkbug-associated Beneficial and Environmental (SBE)” group [11, 13]. Recent studies reported that a different group of *Burkholderia*, belonging to the “plant-associated beneficial and environmental (PBE)” group, is associated with the family Largidae of the superfamily Pyrrhocoroidea [23–25].

Fenitrothion, or *O,O*-dimethyl *O*-(4-nitro-*m*-tolyl) phosphorothioate, which is generally known as “MEP”, is one of the most popular organophosphorus insecticides used worldwide, which inhibits arthropod acetylcholine esterases and exhibits both oral and percutaneous arthropod-specific toxicities [26]. Previous studies have repeatedly isolated MEP-degrading *Pseudomonas*, *Flavobacterium* (reclassified as *Shingobium* [27]), *Cupriavidus*, *Corynebacterium*, *Arthrobacter*, *Sphingomonas*, *Pandoraea*, *Dyella*, *Achromobacter*, *Ralstonia*, and *Burkholderia* from agricultural

field soils [28–32]. These bacteria are able to hydrolyze fenitrothion into 3-methyl-4-nitrophenol and dimethyl thiophosphate, compounds with little insecticidal activity, and metabolize the degradation product as a carbon source for their growth [33]. Although MEP-degraders are not generally observed in untreated soil (or are under the detection limit), intense applications of MEP to soils drastically enrich the MEP-degrading bacteria in the soil [29, 31]. Indeed, enrichment of MEP-degraders in soil was recently observed in natural sugarcane fields in Southern islands of Japan [34], where MEP has been regularly used to prevent sugarcane pests. During the enrichment, the community structure of MEP-degrading bacteria dynamically changes, depending on their MEP-assimilation abilities and the MEP-spraying frequency [35].

Stinkbugs specifically select SBE- or PBE-group *Burkholderia* [11, 25], by use of a sophisticated symbiont-sorting mechanism developed in the midgut [36]. Hence we assumed that transmission of the MEP-degrading *Burkholderia* from the soil to stinkbugs is a two-step selection process [35]: first, the selective growth of specific free-living *Burkholderia* symbionts that can degrade MEP as a carbon source, and thereby modifying the community structure of *Burkholderia* species/strains in the soil; and second, the successful infection of such *Burkholderia* symbiont variants within the host stinkbugs. Previous studies have focused only on the first adaptation step that occurs in soil bacteria in the soil environment [29, 31, 35], whereas because of the lack of stinkbug rearing experiments

on sprayed soil, it is not known how the first and second selection steps are connected. Thus, we currently have no knowledge of how alteration of soil bacterial communities in response to MEP spraying influences the infection frequency of MEP-degrading symbionts in the host stinkbugs. To determine the developing process of the symbiont-mediated insecticide resistance, we investigate soil bacterial dynamics and infected gut symbionts in parallel under different MEP-spraying conditions by culture-dependent and -independent analyses, in conjunction with stinkbug rearing experiments.

Materials and methods

Insecticide-spraying experiments

We collected soil from an agricultural field on which MEP has been sprayed frequently for at least 4 years prior to collection (Supplementary Information). The experimental design of the insecticide-spraying test is shown in Fig. 1. Approximately 150 g (dry weight) of the sieved soil was transferred into plastic pots (10 × 8 cm; opening diameter × depth). Each potted-soil was sprayed once a week with MEP either for 2 weeks (i.e., two-times spraying), four weeks (i.e., four-times spraying), or 6 weeks (i.e., six-times spraying), and the resulting soils were designated as two-times-sprayed soil (2S), four-times-sprayed soil (4S) and six-times-sprayed soil (6S), respectively. As a control, untreated soil, or 0-time-sprayed soil (0S) was investigated. Each spraying experiment was performed in triplicate. MEP spraying treatment, pot maintenance, and soil samplings were performed as previously described ([29]; Supplementary Information).

Culture-dependent and -independent analyses of soil microbiota

Density and diversity of MEP-degrading bacteria in soils were examined by culture-dependent analyses; the colony forming units (CFU) counting and taxonomy identification [29]. Additionally, community structure and abundance of soil microbiota were investigated by culture-independent analyses; the deep sequencing and quantitative PCR of bacterial 16S rRNA genes [29]. Full methods are described in Supplementary Information.

Insect rearing experiment on the MEP-sprayed soils

Riptortus pedestris was reared from hatch to adulthood on the soils differentially treated with MEP, i.e., the 0S–6S, and then the infection frequency of MEP-degrading bacteria was determined. The experimental design is shown in

Fig. 1. Each spraying experiment was performed in triplicate. After soil samples were collected from the 0S–6S, soybean plants (*Glycine max*) germinated from briefly sterilized seeds (dipped in 70% ethanol for 5 min) were potted in these soils, on which hatchlings of the bean bug were reared. Each of the pots included three soybean plants and 15 individuals of first instar nymphs, and was supplied with five dry soybean seeds as insect food. The pots were maintained under a long-day regimen (16 h light, 8 h dark) until adulthood of the insects. The rearing time in the pots was ~20–22 days. From each pot, five adults were randomly selected for further analyses.

After adult emergence, the symbiotic organ (crypt-bearing midgut posterior region) was dissected and subjected to a MEP-degradation assay as previously described ([4]; Supplementary Information). MEP-degradation positive samples were divided in half and subjected to further analyses: one part for the culture-dependent analysis and the other for the culture-independent analysis, as described for soil in Supplementary Information.

Infection dynamics of MEP-degrading symbionts in crop fields

The soil-to-insect infection dynamics of MEP-degrading bacteria in crop fields were investigated in sugarcane fields in Minami-Daito Island, Okinawa, Japan (25°50'N, 131°14' E, altitude of 30–40 m). In this island, most of the sugarcane fields have been treated regularly with MEP for years, resulting in natural occurrence of MEP-degrading bacteria in field soil [34], and natural infection of MEP-degrading *Burkholderia* in a sugarcane pest, *Cavelerius saccharivorus* (superfamily Lygaeoidea; family Blissidae), commonly known as “chinch bug” [4]. This species also acquires SBE *Burkholderia* from environment every generation [19]. Chinch bug adults are short-winged and rarely migrate between sugarcane fields [37]. It is accordingly assumed that chinch bugs collected in a given field are highly likely to have grown and acquired symbionts in the same field. Because of its restricted mobility, this species is considered to be an ideal model for investigating the soil-to-insect infection dynamics of MEP-degrading bacteria under natural conditions. Long-winged adults do occasionally emerge under high-density conditions to enable escape from such unfavorable situations [37]. The fields investigated in this study were sprayed with MEP one to nine times during 2010–2013 seasons [34]. Field soils and inhabiting short-wing adults were collected together on seven randomly selected sugarcane fields in May 2013. In each of the fields, surface soil samples were collected and subjected to CFU counting. To reveal infection frequency of MEP-degrading bacteria in the chinch bug populations, the symbiotic organ of specimens was

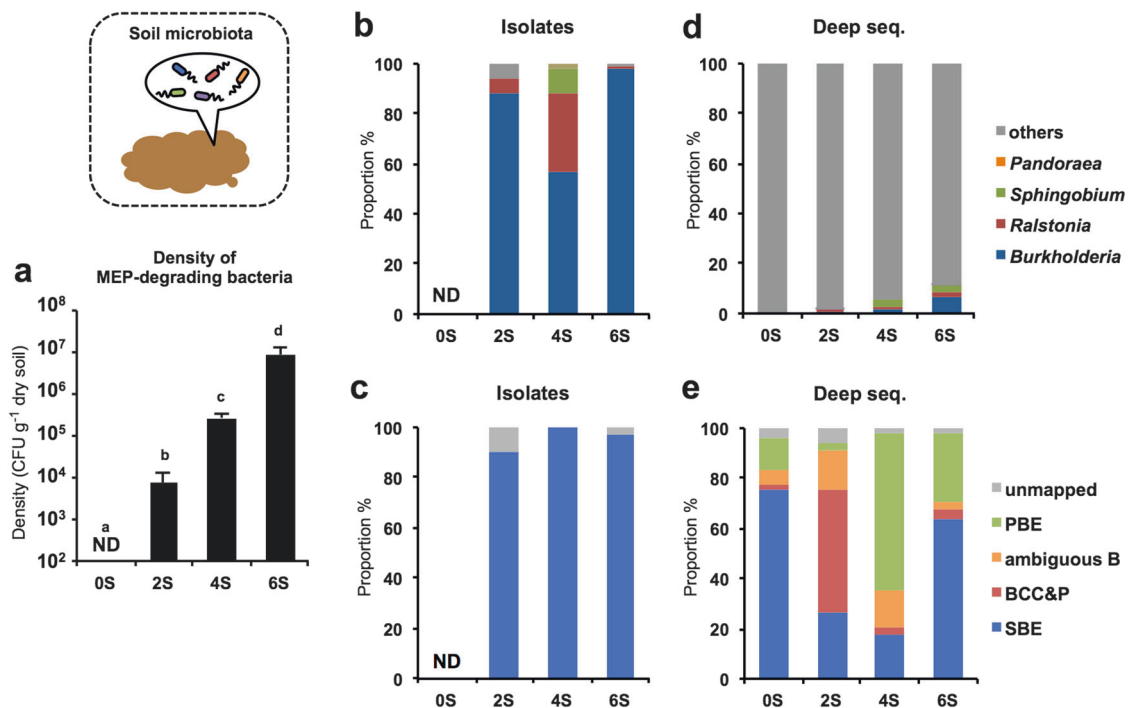


Fig. 2 Population dynamics of MEP-degrading bacteria in sprayed soils. **a** Density of MEP-degrading bacteria in MEP-sprayed soils. Mean $\pm$ SD ($n = 3$) is shown. Values with different letter are significantly difference ($p < 0.05$, ANOVA with Tukey's honestly significant difference test). **b**, **c** Culture dependent analysis of MEP-degrading bacteria isolated from insects. From 2, 4, and 6S soils, respectively 34, 44, and 91 colonies were isolated and identified. **d**, **e**

Culture independent analysis (deep sequencing) of soil microbiota before and after MEP spraying. Genus-level **b**, **d** and *Burkholderia* group-level (**c**, **e**) compositions are shown. Sequences showing $<99.5\%$ identity against reference *Burkholderia* sequences are depicted as "unmapped". Data shown in (**d**) and (**e**) are averages; the corresponding data of all replicates is shown in Supplementary Fig. S5a and b. ND: not detected

dissected and subjected to the MEP-degradation assay (Supplementary Information).

Methods for phylogenetic analysis and statistical analysis are described in Supplementary Information.

Results

Culture-dependent analysis of the population dynamics of MEP-degrading *Burkholderia* in MEP-sprayed soil

Whereas the CFUs of MEP-degrading bacteria were below the detection limit ($<10^2$ cfu g⁻¹ dry soil) in the untreated soil (0S), MEP-degraders were detected after the second treatment with MEP (Fig. 2a). The CFU of MEP-degrading bacteria gradually increased with the MEP-spraying frequency, and reached $8.8 \pm 4.5 \times 10^6$ cfu g⁻¹ soil (mean $\pm$ Standard deviation [SD]; $n = 3$) after the sixth treatment. The MEP-degrading bacteria were identified by sequencing of their 16S rRNA genes, revealing that most of the degraders were of the genus *Burkholderia* (85.2% in total; *Burkholderia* strains/soil-derived isolates in total = 144/169) (Fig. 2b).

The genus *Burkholderia* consists of over 100 species which are classified into three distinct groups in terms of their function and specificity to host organisms [13] (Supplementary Fig. S1a and Table S1). The *Burkholderia cepacia* complex and *B. pseudomallei* (BCC&P) group includes notorious zoonotic species and crop pathogens [38, 39]; the PBE group includes nodule symbionts of some leguminous plants and a number of plant growth promoting species [40]; the SBE group includes a number of species/strains of stinkbug symbionts, as well as the *B. glathei* complex and leaf gall-associated species [41, 42]. These groups correspond to distinct phylogenetic clades within the *Burkholderia* genus (Supplementary Fig. S1a and Table S1). The phylogenetic analysis revealed that most of the MEP-degrading *Burkholderia* isolated from soils belong to SBE (95.8%; SBE strains/*Burkholderia* strains = 138/144) (Fig. 2c), specifically OTU01, 02 and 04 (Supplementary Fig. S2a).

Deep sequencing analysis of the *Burkholderia* population dynamics in MEP-sprayed soil

Principal coordinate analysis (PCoA) indicated that MEP-spraying dramatically changed the community structure of

Table 1 Deep sequencing analysis of soil samples

Experiment ID	Replication ^a	No. of sequences ^b	No. of <i>Burkholderia</i> sequences	Proportion of <i>Burkholderia</i> ^c	Proportion of <i>Burkholderia</i> group % ^d					
					SBE	Ambiguous A	BCC&P	Ambiguous B	PBE	Unmapped ^c
0S	1	91,884	62	0.07	65.6	0	4.9	4.9	18.0	6.6
	2	54,218	30	0.06	76.7	0	0	13.3	3.3	6.7
	3	85,407	60	0.07	76.7	0	1.7	5.0	15.0	1.7
2S	1	93,985	541	0.58	24.5	0	50.9	14.0	3.3	7.5
	2	90,666	514	0.57	23.4	0	52.0	15.3	3.6	5.8
	3	98,302	539	0.55	31.6	0	45.0	15.6	3.4	4.4
4S	1	75,400	1371	1.8	18.7	0	3.0	14.3	62.6	1.3
	2	94,871	1851	2.0	14.3	0	2.6	18.6	61.9	2.7
	3	92,846	1837	2.0	18.8	0	3.1	13.4	62.6	2.2
6S	1	128,961	9014	7.0	66.1	0	3.4	4.1	24.9	1.6
	2	94,365	6424	6.8	65.5	0	4.1	2.1	26.6	1.8
	3	77,321	5330	6.9	61.0	0	4.1	3.0	29.7	2.3

^a Three replications were prepared for each MEP-spraying treatment

^b Number of sequences after removal of low quality, chimeric, and archaeal sequences

^c Proportion of *Burkholderia* sequences to bacterial sequences

^d Proportion of sequences of each *Burkholderia* groups to *Burkholderia* sequences

the soil microbiota (Supplementary Fig. S3a), wherein *Burkholderia*, *Sphingobium*, *Ralstonia*, *Nevskia*, *Methylobacterium*, and *Methylophilus* consistently increased during the spraying (Supplementary Fig. S4), as also reported in our previous study [29]. In relative abundance, *Burkholderia* (i.e., all reads assigned to the genus *Burkholderia*) was only $0.06 \pm 0.01\%$ (mean $\pm$ SD) before treatment, while the amount gradually increased to 0.57 ± 0.01 , 1.92 ± 0.07 , and $6.90 \pm 0.07\%$ after the second, fourth and sixth MEP-treatments, respectively (Fig. 2d, Table 1, Supplementary Fig. S5a). Diversity indices, i.e., Chao1, Shannon and reciprocal Simpson, decreased after MEP-spraying (Supplementary Table S2), confirming that MEP treatment enhanced some specific groups of bacteria including *Burkholderia* and caused the decrease of soil microbial diversity. The qPCR analysis showed that the copy number of bacterial 16S rRNA genes was stable at around $2 \times 10^{10} \text{ g}^{-1}$ -soil during the treatments (Supplementary Table S2). It should be noted here that the deep sequencing analysis demonstrated that the *Burkholderia* fraction in the soil is remarkably small, particularly in the early treatments, although they were abundant in the culture-dependent method (Fig. 2b).

Not unlike the result of the culture-dependent method (Fig. 2c), the deep sequencing analysis revealed that group-level and OTU-level compositions of *Burkholderia* dynamically changed during the treatments (Fig. 2e, Supplementary Fig. S5b): OTU15 in BCC&P firstly grew but then decreased until the fourth MEP-treatment; OTU25 in PBE

dominated after the fourth treatment; and eventually OTU01 in SBE became dominant after the sixth treatment with MEP.

Bean bugs acquire MEP-degrading *Burkholderia* when reared on MEP-treated soils

Since the symbiotic organ of infected (symbiotic) insects is readily distinguishable from uninfected (aposymbiotic) ones by visual inspection [43] and by PCR amplification for bacterial 16S rRNA genes, the dissection of the insects and diagnostic PCR revealed that each individual from all soil samples was symbiotic (see below). The infection frequency of MEP-degrading bacteria was investigated by inspecting MEP-degrading activity of the dissected symbiotic organs using a spectrophotometric MEP-degradation assay. The result demonstrated that the infection frequency of MEP-degrading bacteria in the bean bug gradually increased depending on the frequency of MEP-spraying: bean bugs reared on untreated soils (0S) were all negative, while 33.3 ± 18.9 , 66.7 ± 24.9 , and $86.7 \pm 9.4\%$ (mean $\pm$ SD; $n = 3$) of insects reared on the two-, four-, and six-time-treated soils (2S, 4S, and 6S), respectively, were MEP-degradation positive (Fig. 3a).

In a culture-dependent analysis of MEP-degradation positive insects, all isolated strains showed the MEP-degrading activity. They were subjected to direct sequencing of 16S rRNA genes for identification. In total, 260 colonies of MEP-degrading bacteria were isolated from 28

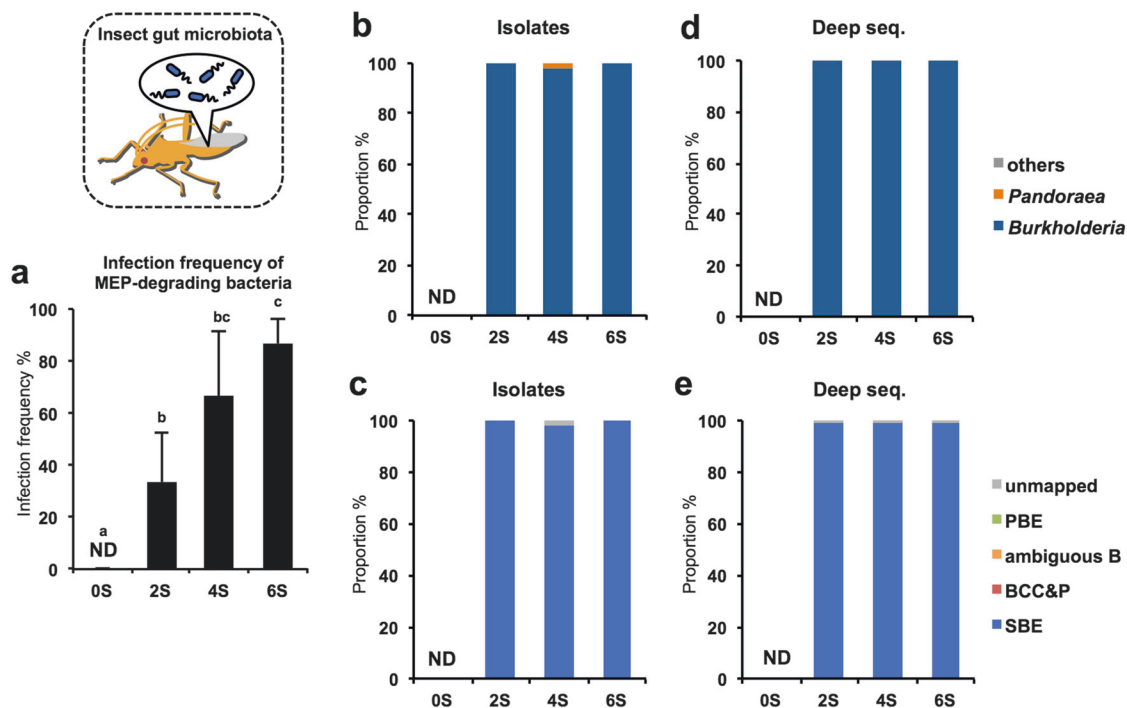


Fig. 3 Population dynamics of MEP-degrading bacteria in bean bug reared on MEP-sprayed soils. **a** Infection frequency of MEP-degrading bacteria in bean bugs when the insects were reared on MEP-sprayed soils. Five insects per soybean pot were subjected to a MEP-degradation assay. Mean $\pm$ SD ($n = 3$) is shown. Values with different letter are significantly difference ($p > 0.05$, ANOVA with Tukey's honestly significant difference test). **b, c** Culture dependent analysis of MEP-degrading bacteria isolated from insects. From insects reared on

2, 4, and 6S pods, 47, 91, and 122 colonies were isolated and identified, respectively. **d, e** Culture independent analysis (deep sequencing) of gut microbiota of insects infected with MEP-degrading bacteria. Genus-level **b, d** and *Burkholderia* group-level (**c, e**) compositions are shown. Sequences showing $<99.5\%$ identity against reference *Burkholderia* sequences are depicted as "unmapped". Data shown in **d, e** are averages; the corresponding data of all replicates is shown in Supplementary Fig. S5c and d. ND: not detected

insects with 99.2% (258 colonies) belonging to the genus *Burkholderia* (Fig. 3b). Almost all of these MEP-degrading *Burkholderia* strains were placed into the SBE group (Fig. 3c). In total, 37 OTUs of *Burkholderia* were detected by clustering of reference sequences shown in Supplementary Table S1, among which OTU01 was by far the most prevalent throughout the rearing experiments (96.4% infection frequency; insects infected with OTU01/total insects infected with any MEP-degraders = 27/28) (Supplementary Fig. S2b).

Deep sequencing analysis of the symbiotic organs with MEP-degrading activity confirmed the extremely low-bacterial diversity and the predominance of SBE *Burkholderia* (Fig. 3d, e, Table 2, Supplementary Fig. S5c and d), which was different from soil microbiota (Supplementary Fig. S3b). Particularly, OTU01 of the SBE group was the most predominant phylotype in insects showing a MEP-degrading activity (Fig. 4), confirming the results of the culture-dependent analysis (Supplementary Fig. S2b). Although the sequenced region was limited to 255 bp, this sequence information was sufficient to distinguish the SBE, BCC&P and PBE clades (Supplementary Table S1). The deep sequencing data in conjunction with the culture-based

results demonstrated that microbiota of the gut symbiotic organ with MEP-degrading activity was extremely simple, occupied with only a few OTUs of the SBE group.

Infection dynamics of MEP-degrading *Burkholderia* from soil to insect

Although *Burkholderia* dominated in both soil and insect, the population dynamics of the MEP-degrading *Burkholderia* showed a striking contrast between them; the OTU composition in the *Burkholderia* population dramatically changed in the MEP-sprayed soil, while the OTU associated with bean bugs was remarkably simple and stable during the series of experiments (Fig. 4a, b: compare Figs. 2e, 3e at the OTU level). The OTU-level comparison revealed OTU01 was the major MEP-degrading symbiont of bean bugs. Deep sequencing analysis revealed that relative abundance of OTU01 was extremely small, $0.004 \pm 0.002\%$ of the total analyzed bacterial population, in untreated soil, wherein the CFU of MEP-degrading bacteria was under the detection limit ($<1.0 \times 10^2$ cfu g⁻¹-soil). Relative abundance of the OTU01 was still very small, only $0.04 \pm 0.01\%$, in the two-times-treated soil (2S); while the

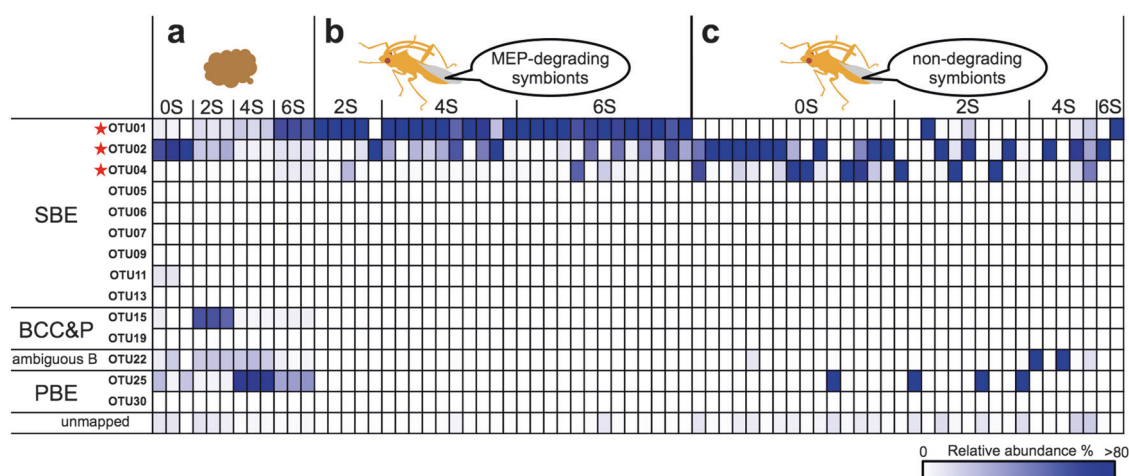


Fig. 4 Dynamics of *Burkholderia* in MEP-sprayed soils and reared insects. *Burkholderia* OTUs in soils (a), in insects showing a MEP-degrading activity (b), and in insects showing no MEP-degrading activity (c). Based on deep sequencing analyses, *Burkholderia* dynamics in soils and insect-associated *Burkholderia* were compared in a heatmap. Within the currently identified *Burkholderia* species, 37 possible OTUs in total were generated by clustering the V4 region (255 bp) of 16S rRNA genes (>99.5% sequence identity). Of these 37

OTUs, 14 OTUs that include more than one mapped sequence were detected in this study. Sequences showing <99.5% identity are depicted as unmapped. A color gradient shows relative abundance of OTUs in a *Burkholderia* population within each sample. Stars indicate OTUs including MEP-degrading strains isolated from both insects and soils, among which OTU01 was the most abundant (see also Supplementary Fig. S2)

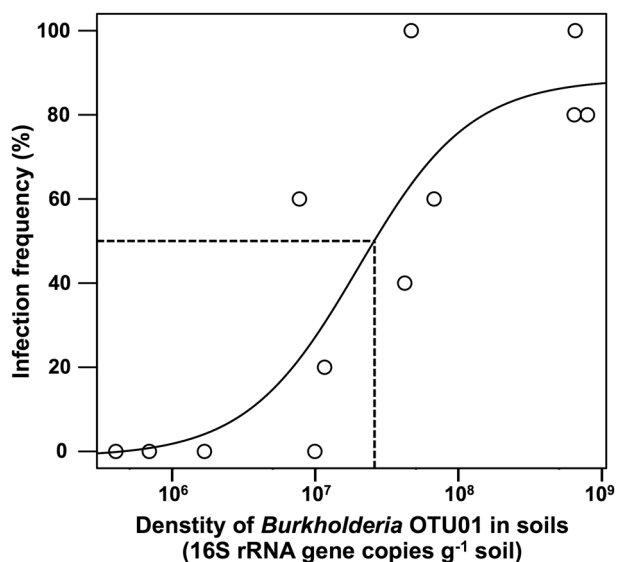


Fig. 5 Infective dose of a MEP-degrading *Burkholderia* (OTU01) in *R. pedestris*. The infection frequency of *Burkholderia* OTU01 in insects and the environmental density of it in soils were used to estimate the 50% infective dose (ID₅₀) of the *Burkholderia*. The density of *Burkholderia* OTU01 was estimated by the copy number of bacterial 16S rRNA gene, and the relative abundance of OTU01 was determined by deep sequencing. Dotted lines indicate ID₅₀ of *Burkholderia* OTU01

abundance of it increased to $0.24 \pm 0.03\%$ in the four-time-treated soil (4S) and eventually reached $3.49 \pm 0.16\%$ in the six-time-treated soil (6S).

A notable conclusion of this data is that since insects carrying the OTU01 MEP-degrading symbiont appeared

already after two-times spraying with MEP, the insect can acquire a MEP-degrading SBE (i.e., OTU01) from the ambient soil in which it is only present as a small fraction, $0.04 \pm 0.01\%$ of the bacterial population. The actual number of bacterial cells remains unknown, but considering the qPCR results of bacterial 16S rRNA genes (Supplementary Table S1) and deep sequencing results of the proportion of *Burkholderia* OTU01 in soil microbiota, the estimated copies number of 16S rRNA genes of the OTU01 was $9.7 \pm 1.6 \times 10^6$ copies per g soil in 2S, $5.2 \pm 1.1 \times 10^7$ copies per g soil in 4S, and $6.9 \pm 0.7 \times 10^8$ copies g⁻¹ soil in 6S. Together with the results of infection frequency of insects to *Burkholderia* OTU01 (Fig. 4), the 50% infective dose (ID₅₀), defined as the amount of MEP-degrading *Burkholderia* OTU01 in soils required for colonization of 50% of tested insects, was estimated by sigmoid curve fitting as 2.0×10^7 copies per g soil (Fig. 5).

Infection dynamics of non-MEP-degrading *Burkholderia* in bean bugs

Apart from the insects infected with MEP-degrading symbionts, the other individuals were infected with non-MEP-degrading *Burkholderia* (Supplementary Fig. S6). In contrast to the infection frequency of MEP-degrading *Burkholderia*, infection frequency of non-degraders gradually decreased depending on the MEP-spraying (Supplementary Fig. S6a). In total, 32 insects infected with non-degraders were subjected to deep sequencing analysis. *Burkholderia* was detected from all of the 32 individuals (Supplementary

Fig. S6b), although *Pandora*, a sister group of the genus *Burkholderia*, was predominant in three individuals. The non-degrading *Burkholderia* were mostly SBE, while some PBE- and ambiguous B-group *Burkholderia* were frequently detected in four and two individuals, respectively, (Supplementary Fig. S6). At the OTU level, OTU02 and OTU04 of the SBE group were predominant phylotypes in the insects infected with non-degrading *Burkholderia* (Fig. 4c). By contrast to the MEP-degrading symbiont OTU01, the OTU02 was detected frequently in the untreated soil (0S) but decreased after MEP-spraying (Fig. 4a), which corresponds to the gradual reduction of infection frequency of non-degrading *Burkholderia* in bean bugs (Supplementary Fig. S6a).

In this study, OTUs were assigned by only 255 bp sequences of 16S rRNA gene, which is the current technological limit in deep sequencing analysis. With such restricted information, genetically and phenotypically different strains could be assigned to the same OTU (Supplementary Table S1). In addition, since MEP-degrading genes are often encoded on a plasmid [33, 44–46], the MEP-degrading trait could be lost or gained via plasmid loss or transfer. In this context, it can be expected that genetically similar (or identical) phylotypes show different MEP-degrading activities. OTU02 and OTU04 were rare in the insects infected with MEP-degrading *Burkholderia* but predominant in insects infected with non-MEP-degrading

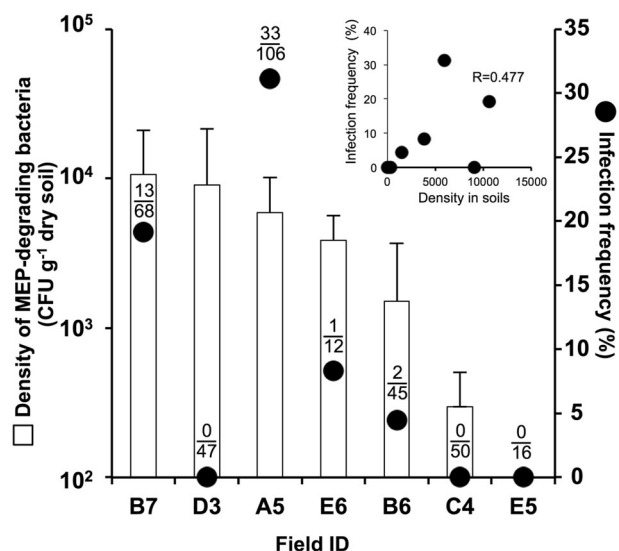


Fig. 6 Infection dynamics of MEP-degrading bacteria from soil to insect in sugarcane fields. Closed circles indicate the infection frequency of the sugarcane pest bug, *Cavalerius saccharivorus*, with MEP-degrading bacteria. Numbers of infected insects/total insects investigated are depicted above the circle. Open bars indicate the CFU of MEP-degrading bacteria in field soils (referred from [34]). The inset shows the correlation analysis between the density of MEP-degrading bacteria and the infection frequency of insects with MEP-degrading bacteria ($R = 0.477$, $p = 0.279$)

Table 2 Summary of deep sequencing analysis of insects' gut samples

Experiment ID	MEP-degrading activity	No. of insects	No. of total sequences	Proportion of <i>Burkholderia</i> group % ^b				
				SBE	ambiguous A	BCC&P	Ambiguous B	PBE
0S	Positive	0	—	—	—	—	—	—
2S	Negative	15	28,452 ± 9387	89.7 ± 24.0	0	0	0.5 ± 1.7	6.1 ± 22.6
	Positive	5	30,447 ± 6347	99.5 ± 0.25	0	0	0.004 ± 0.002	0
4S	Negative	10	18,117 ± 10,865	69.0 ± 43.6	0	0	0.40 ± 0.54	26.6 ± 40.7
	Positive	10	29,331 ± 28,698	99.0 ± 1.13	0	0	0.11 ± 0.32	0.007 ± 0.02
6S	Negative	5	14,018 ± 8933	53.1 ± 43.3	0	0	41.5 ± 47.1	0.06 ± 0.10
	Positive	13	26,123 ± 7899	98.9 ± 1.62	0	0	0.005 ± 0.01	0.001 ± 0.003
	Negative	2	34,442 ± 12,105	99.3 ± 0.53	0	0	0.004 ± 0.0001	0
	Positive	2	—	—	—	—	—	—

^aRatio of no. of *Burkholderia* sequences per no. of total sequences. The assignment was performed by the RDP classifier with a 80% confidence threshold

^bRatio of no. of each group per no. of *Burkholderia* sequences. The assignment was performed by BLAST-N search with >99.5% identity cutoff

^cSequences not assigned as any *Burkholderia* groups.

Burkholderia (Fig. 4), suggesting that the majority of OTU02 and OTU04 are non-degrading. Similarly, culture-dependent and -independent results strongly suggested that the majority of OTU01 is MEP-degrading (Fig. 4; Supplementary Fig. S2).

Infection dynamics of MEP-degrading bacteria in crop fields

The soil-to-insect infection dynamics of MEP-degrading bacteria in crop fields were investigated in sugarcane fields in Minami-Daito Island. The CFU of MEP-degrading bacteria in field soils [34] and the infection frequency of MEP-degrading bacteria in field-collected *C. saccharivorus* (short-wing adults) were comparatively analyzed. The results were not entirely consistent but nevertheless showed a tendency of insects living on a soil inhabited by a high density of MEP-degraders being highly infected with MEP-degrading symbionts (Fig. 6).

Discussion

We investigated how MEP spraying affected the dynamics of MEP-degrading bacteria in the soil and how this influenced the infection and acquisition of MEP-degrading symbionts by stinkbug hosts. Our study demonstrated that: (1) diverse strains of MEP-degrading *Burkholderia* dynamically change in soils depending on MEP-spraying frequency (Figs. 2e, 4); (2) among the diverse strains of MEP-degrading *Burkholderia*, a particular phylotype of the SBE group (OTU01) is nearly exclusively acquired by the bean bug (Fig. 4, Supplementary Fig. S2); (3) the specific infection and acquisition can occur after only two-times MEP spraying even though the relative abundance of the MEP-degrading phylotype is only $0.04 \pm 0.01\%$ in soil (estimated ID_{50} : 2.0×10^7 copies of 16S rRNA genes g^{-1} soil) (Figs. 3, 4, 5); (4) as the abundance of the degrading strains increases in soil by spraying, the infection frequency of the MEP-degrading phylotype increases (Figs. 2a, 3a); and therefore, (5) the infection frequency of the MEP-degrading phylotype strictly depends on MEP-spraying and the resulting enrichment of the MEP-degrading symbiont in the soil. Furthermore, (6) the investigation of natural populations of a chinch bug strongly suggested that the density-dependent acquisition of MEP-degrading *Burkholderia* occurs in agricultural fields (Fig. 6). Population dynamics and succession of *Burkholderia* in soils after MEP spraying have been repeatedly studied by both culture-dependent and -independent approaches [29, 31, 34, 35, 47]; however, no study has investigated how such bacterial dynamics in soil under MEP-spraying condition influences the development of symbiont-mediated MEP

resistance. This is, to our knowledge, the first clear-cut study demonstrating how environmental dynamics of bacteria affects the development of a symbiont-mediated and agriculturally important insect trait, insecticide resistance.

The evolution of insecticide resistance is generally considered to involve the modification of a pest's own phenotype, in which the emergence of a resistance trait via mutation and/or rearrangement of the pest's genome is selected by repeated application of the insecticide. The evolution of insecticide resistance is rather fast, but still requires several pest generations, even in the most rapid cases [48, 49]. Notably, infection with the MEP-degrading *Burkholderia* has been detected within 2 weeks after only two sprayings with MEP (Fig. 3a), suggesting that, via the intermediacy of MEP-degrading symbionts, insecticide resistance could evolve in a pest bug population more rapidly than was previously thought. This study has demonstrated that the symbiont-mediated insecticide resistance develops through two selection steps, i.e., selective growth of insecticide-degrading bacteria upon spraying and selective acquisition of symbionts by host insects, the development of which strongly depends on the first step occurring in bacteria in the soil. The rapid emergence of symbiont-mediated insecticide resistance after spraying could therefore be explained by the rapid evolution of soil bacteria.

In this study, two different stinkbug systems were investigated. In the laboratory experiments performed on the bean bug, as the density of MEP-degrading bacteria in soil increased from $<10^2$ to 10^4 cfu g^{-1} soil by two-times spraying of MEP, the infection frequency of MEP-degrading *Burkholderia* increased from 0 to $33.3 \pm 18.9\%$ (Figs. 2a, 3a). In the field survey of the chinch bug, in which the density of MEP-degrading bacteria in the soil ranged from 10^2 to 10^4 cfu g^{-1} soil, the infection frequency of MEP-degrading bacteria varied from 0 to 31.1% (Fig. 6). Despite the different systems, the similar soil-to-insect dynamics of MEP-degrading bacteria was observed, strongly suggesting that the relatively rapid evolution of insecticide resistance that we revealed by the laboratory experiments could also occur in natural crop fields. Based on these values, we here estimate that 10^2 cfu g^{-1} soil of MEP-degrading bacteria is a warning value for the emergence of symbiont-mediated insecticide resistance.

The enrichment of MEP-degrading bacteria in soils followed by the emergence of infected insects is most likely influenced by the spraying history of the insecticide. Our previous study revealed that MEP-degrading bacteria increase in response to MEP-spraying more quickly in soils with MEP-spraying history than in those without spraying history [29]. In such naive soils, infection of insects with MEP-degrading symbionts would be rare because of the low density of degrading symbionts present under the

infection threshold, even after multiple sprayings. In contrast, sugarcane fields in Minami-Daito Island have been consistently treated with MEP and similar organophosphorus pesticides over 30 years. MEP-degrading bacteria naturally inhabit these soils and can be detected without the enrichment [34]. In such intensely-treated soils, insects could be infected with MEP-degrading bacteria without any additional spraying (Fig. 6), which illustrates in an agronomic setting the risk of insecticide resistance through the acquisition of symbionts.

The soil-to-insect infection dynamics of MEP-degrading *Burkholderia* (Fig. 4) indicates the remarkably high host-symbiont affinity between the bean bug and SBE group of *Burkholderia*. Our previous study revealed that *R. pedestris* acquires the *Burkholderia* symbiont extremely efficiently from the environment, with an ID₅₀ of only 80 symbiont cells in rearing experiments [14]. Recently, we described a specific gut organ for bacteria sorting, the so-called “constricted region” in the insect’s midgut [36], by which the *Burkholderia* symbiont is exclusively selected from the enormously diverse soil microbiota. Although it remains unclear how specific the constricted region is in bacterial selection, it is plausible that this sorting mechanism is involved in the infection dynamics of MEP-degrading *Burkholderia*. Moreover, several bacterial adaptations have been identified that are essential for or contribute to the colonization of the symbiotic organ [36, 50–53], which should be involved in the specific and efficient acquisition of MEP-degrading *Burkholderia*.

This study analyzed the soil-to-insect infection process of MEP-degrading *Burkholderia*; then, is the reverse transmission of these MEP-degrading *Burkholderia* from insect to soil possible? Although it remains unclear whether the *Burkholderia* symbiont is released from the stinkbug host, the reverse transmission has been reported in other symbiotic systems with environmental transmission, such as the legume-*Rhizobium* and squid-*Vibrio* symbioses [54–56]. The release of MEP-degrading *Burkholderia* from stinkbugs would be of primordial importance in the development of symbiont-mediated insecticide resistance for the following two points. First, such infection-proliferation-escape process could increase the symbiont density in the environment, leading to high-infection fidelity. Second, since stinkbugs move actively from field to field, release of MEP-degrading symbionts from infected insects could lead to the spread of resistant insect metapopulations to fields without MEP-spraying history.

Conclusion

The arms race between pest insects and human invention of insecticides has intensified and has been a serious concern

through our agricultural history, and in some cases remarkably rapid evolution of insecticide-resistance has been reported [57]. The rapid evolution of insect traits is generally thought to be determined by the insect’s genome, and the evolutionary process has been described only through the perspective of the insect’s population dynamics and genetics, while until now, symbiosis has not been considered. Diverse insect traits, such as food digestion, sex, heat tolerance and even body color, can be affected by symbiotic microorganisms [58–61]. Plants and soil on which insects inhabit are populated with an enormous quantity and diversity of microorganisms [62]. Hence, as shown here, environmental microorganisms could play a pivotal role in insect physiology, ecology, and evolution, as tightly associated microbiota do so. Now we should focus more on environmental microbial ecology to comprehensively understand and manage pest insects.

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Author contributions H.I. and Y.K. designed the project. H.I. performed the insect rearing experiment. H.I., A.N., K.T., M.H., Y.S., and Y.K. collected the soil and insect samples. H.I., K.T., and M.H. conducted the culture dependent analysis. H.I., T.H., and Y.S. performed the deep sequencing analysis. H.I. analyzed the results. H.I. and Y.K. wrote the manuscript. All co-authors edited the manuscript before submission.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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