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The fall armyworm converts maize endophytes into its own probiotics to detoxify benzoxazinoids and promote caterpillar growth

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Abstract

Background The fall armyworm (FAW, *Spodoptera frugiperda*) threatens maize production worldwide, and benzoxazinoids (Bxs) are known as the main secondary metabolites produced by maize to defend against FAW. However, we do not yet know whether and in what ways certain endophytes in the digestive system of FAW can metabolize Bxs, thus enhancing the fitness of FAW when feeding on maize.

Results Using Bxs as the sole carbon and nitrogen source, we isolated *Pantoea dispersa* from the guts of FAW. *P. dispersa* can colonize maize roots and leaves as indicated by GFP-labeling and further successfully established itself as an endophyte in the Malpighian tubules and the gut of FAW after FAW feeding activities. Once established, it can be vertically transmitted through FAW eggs, suggesting the potential that FAW can convert maize-derived endophytes into symbiotic bacteria for intergenerational transmission. The prevalence of *P. dispersa* in FAW guts and maize leaves was also confirmed over large geographic regions, indicating its evolutionary adaptation in fields. Bxs determination in the gut and frass of FAW combined with bioassays performance on maize *bx2* mutants revealed that the colonization of *P. dispersa* can promote FAW growth by metabolizing Bxs rather than other metabolites. Additionally, genome and transcriptome analyses identified plasmid-borne genes, rather than chromosomes of this species, were crucial for Bxs metabolism. This was further validated through in vitro prokaryotic expression assays by expressing two candidate genes form the plasmid.

Conclusions FAW can convert maize endophytes into its own probiotics to detoxify Bxs and thus enhance caterpillar growth. This represents a novel strategy for lepidopteran pests—transforming allies of the host into its own—thereby shedding light on the rapid spread of FAW and enhancing our understanding of ecological and evolutionary mechanisms underlying the pest-microbe–plant interactions.

Keywords *Spodoptera frugiperda*, Maize, Benzoxazinoids, *Pantoea dispersa*, Pest-microbe–plant interactions

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Background

Spodoptera frugiperda, known as fall armyworm (FAW), is a lepidopteran pest that feeds on more than 350 plant species, causing major damage to numerous crops, including maize. FAW was first reported in the USA in 1876 and was recently found in Europe and Africa (2016), India (2018), and China (2019) [1]. It can complete several generations within a single breeding season, evolves rapidly, and is becoming an endemic pest worldwide. Just 1 year after it was first reported in China, FAW was listed as a class I crop pest that severely threatens maize production.

Although plants, including maize, cannot move to avoid pests, the course of millions of years of natural evolution and long-term breeding and domestication has led maize to develop complex and effective defense strategies against pests. Maize can recognize elicitors in the oral secretions of pests and subsequently initiate a series of defense responses, including direct and indirect defense responses [2, 3]. The direct defense responses include the accumulation of certain secondary metabolites in maize tissues upon insect infestation. These secondary metabolites can poison or inhibit the digestion and absorption processes of insects and inhibit their growth [2]. The most well-studied secondary metabolites against insect herbivores in maize are benzoxazinoids (Bxs, including hydroxamic acid and its derivatives), which are broad-spectrum compounds directly involved in the anti-insect response in maize and can even act as natural insecticides [4]. Numerous studies have shown that Bxs are effective at promoting the resistance of maize to many pests, including aphids (*Rhopalosiphum maidis*) [5], corn borers (*Ostrinia furnacalis*) [6], *Spodoptera litura*, and FAW [7]. In contrast to other pests, however, FAW can reglycosylate DIMBOA (a major compound of Bxs) to form DIMBOA-Glc, which is subsequently excreted through feces to reduce its toxicity. However, FAW cannot reglycosylate HDMBOA; therefore, HDMBOA retains its toxicity to FAW and therefore serves as a pivotal indicator of Bx compounds for the control of FAW [7].

The battle between plants and their insect pests has lasted approximately 400 million years [8]. Current studies in the plant–herbivore interaction field have focused mainly on the accumulation of plant secondary metabolites against herbivores and the functions of detoxification enzymes, such as carboxylesterases (CarEs) [9], glutathione S-transferases (GSTs), and cytochrome P450s (P450s), in the insect gut [10]. However, little attention has been given to investigating whether microorganisms help plants defend against pests or aid pests in detoxifying and metabolizing relevant anti-insect secondary metabolites.

Microbial–plant, microbial–microbe, and microbial–insect interactions have been extensively documented. Microorganisms are active participants in a wide range of physiological processes in plants, with the most well-known example being the nitrogen-fixing bacteria in legumes. These bacteria can convert atmospheric nitrogen into nitrates, which are readily accessible for plant utilization [11]. Nitrogen-fixing bacteria, such as *Pantoea* and *Klebsiella* spp., have also been reported to cooperate with fungi and convert atmospheric nitrogen into available compounds as fertilizers that fungi can use, facilitating the growth of fungi and providing food for ants [12]. It was also found that when endophytic bacteria including *Pantoea dispersa* are removed from the gut of a stinkbug (*Plautia stali*), the mortality rate of *P. stali* increases significantly, and reinoculation with endophytes restored the normal growth of *P. stali* [13]. Microbes were also found to be involved in the digestion of plant metabolites within the insect gut. For instance, the sap-sucking beetle *Cassida rubiginosa* relies on the pectinase secreted by its endophyte *Stammera* to decompose pectin present in the plant cell wall [14]. *Stammera* can be vertically transferred to the next generation of beetles via their eggs. Certain gut microbes can also help insects grow and survive by detoxifying plant-specific secondary metabolites. For instance, some species in the gut microbiota of the coffee berry borer (*Hypothenemus hampei*) subsist entirely on caffeine as a sole carbon and nitrogen source, and this detoxification of caffeine is essential for the survival of *H. hampei* [15].

Despite the studies mentioned above, current research on the triadic interactions between herbivores, microbes, and plants remains limited. We wondered whether and how certain microbes are involved in the battle between maize and FAW. This presents a clear gap in the field of plant–herbivore resistance research, especially in regard to economic crops. In this study, we aimed to determine whether FAW can convert maize endophytes into its own endophytes when feeding on maize leaves and whether the acquired species after conversion can metabolize Bxs, helping FAW detoxify these compounds and promote caterpillar growth. Elucidating the underlying mechanism may help to improve our understanding of the rapid worldwide spread of this devastating maize pest. Here, we isolated a well-reported environmental microbe, *P. dispersa*, which was initially an endophyte of maize but was able to establish itself within the Malpighian tubules of FAW and metabolize Bxs during FAW feeding and promote FAW growth. Our finding of a single species of microbe acquired by FAW when feeding on maize and consuming Bxs as a substrate sheds light on the mechanisms underlying the spread of these major pests among

crops and provides a new perspective on plant–microbe–herbivore interactions.

Methods

For detailed information on the materials and methods, please refer to supplementary note 1.

Separation and purification of Bxs

Bxs separation and purification were performed through the Amberlite XAD-7-mediated ion exchange method modified according to previous methods [16].

Screening of Bx-metabolizing *bacteria*

Bacteria from the gut of field-collected 5th instar FAW larvae were screened by utilizing Bxs as the sole carbon and nitrogen source via 7 rounds of selection. The ability of the screened bacteria on Bxs metabolism was determined on an LC–MS system (LCMS8040, Shimazu) [17].

GFP labeling and *P. dispersa* location imaging

The colonization of both maize and FAW by *P. dispersa* was tracked by utilizing the green fluorescent protein (GFP) gene [18, 19]-transformed *P. dispersa*. All images were taken with a motorized fluorescence stereomicroscope (SMZ-18, Nikon) equipped with a 13.5 × objective lens (Nikon) as well as a research microscope equipped with fluorescence filters and a light source (Leica DM5500 B).

Aseptic insect handling and inoculation with symbionts

Aseptic insects were generated by egg sterilization, after which gentamicin, streptomycin, rifampicin, and tetracycline were added to the diet. Herbivore performance was indicated by the final larval mass as previously reported [17].

Determination of nitrogen fixation

The nitrogen fixation activity of the isolated *P. dispersa* was measured based on acetylene reduction methods following a previous study [12].

Collection of field samples, preparation of DNA and qPCR

The sampling sites spanned the geographical coordinates of 98.495 E to 112.332 E and 18.386 N to 24.755 N (Fig. S1) during the FAW outbreak season (August 2021 to August 2023). The absolute abundance of *P. dispersa* in field-collected maize leaves, FAW and two other greenhouse-raised maize pests (*Mythimna separata* and *Spodoptera litura*), was determined by adding known number of *Escherichia coli* cells harboring a plasmid containing an artificial DNA fragment before DNA extraction as an external standard according to our previous study [20].

16S rRNA and genome sequencing

16S rRNA and genome sequencing were performed on the PacBio Sequel platform at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). The detailed data analysis included adapter removal [21], assembly of the filtered sequencing data [22], tRNA prediction [23], and annotation of protein-coding genes as previously described [24].

Transcriptome analysis of *P. dispersa*

P. dispersa grown in mineral media (Con) or mineral media supplemented with Bxs were sequenced on an Illumina platform by Shanghai Personal Biotechnology Co., Ltd. After filtering out low-quality reads [21], HTSeq 0.6.1p2 and DESeq (version 1.18.0) were used to identify differentially expressed genes (DEGs) [24].

Enzyme activity experiment

The target gene IDs and primers used are listed in Table S1. The PCR products were ligated into the pet32a + vector and subsequently introduced into the BL21 strain. The protein of target gene was induced via 1 mM IPTG and purified with a His-tag Protein Purification Kit (Beyotime Biotechnology) and confirmed via western blotting with a His-tag antibody (Abmart) to confirm full translation of the target genes. Bxs levels were determined via LC–MS and compared between BL21 strains expressing the pet32a + empty vector and those infused with target genes or among the abovementioned purified proteins in 25 mM Hepes buffer (pH 7.0) as previously reported [25].

Results

Isolation and identification of microorganisms that metabolize Bxs

We first examined the growth of FAW in the presence of natural gut microbiome through the metabolism of Bxs. By feeding normal and aseptic FAW larva on surface-sterilized maize leaves, we found that the normal FAW gained significantly more weight than the aseptic FAW (Fig. 1A), and when these FAW feeding on the artificial diet with or without Bxs, the aseptic FAW gained less weight only when feeding on diets with Bxs (Fig. 1B), indicating the overall microbiome in the gut of FAW (Fig. 1C) can assist FAW growth by coping with Bxs. These results inspired us to further explore certain bacterial species in gut of FAW that can metabolite Bxs, which is a group of compounds containing carbon and nitrogen [26]. We used gradient dilution (for selecting out microorganisms cannot metabolize Bxs) and enrichment cultivation (for propagating microorganisms metabolize Bxs) to isolate FAW endophytes capable of using Bxs as

carbon and nitrogen source and sequenced 32 isolates that grew on agar plates (Fig. 1D), which included *P. dispersa*, *Klebsiella variicola*, *Brucella cicero*, *Brucella intermedia*, and *Ochrobactrum* sp. After reconfirmation with liquid media supplemented with Bxs as the sole carbon and/or nitrogen source, we found that the only strain that had a significant effect on the metabolism of Bxs was *P. dispersa*. When Bxs served as the sole carbon source (indicated as plus N [nitrogen]), the remaining levels of DIMBOA, DIMBOA-Glc, HDMBOA-Glc, and HMBOA-Glc were 62.5%, 43.0%, 58.0%, and 34.2%, respectively, after 3 h of metabolization by *P. dispersa*. Moreover, when Bxs served as the sole nitrogen source (indicated as plus C [carbon]) or served as both a nitrogen and carbon source (indicated as No CN, Fig. 1E–H), the levels of remaining Bxs after 3 h of metabolism by *P. dispersa* were also significantly lower than those in the controls (with a medium containing an equivalent amount of *P. dispersa* that was rendered inactive at 65 °C for 30 min, accounting for any potential adsorption effects of bacterial cells on Bxs). These data indicate that *P. dispersa* can use Bxs as both a carbon and a nitrogen source.

Colonization of *P. dispersa* in maize and FAW

To investigate whether *P. dispersa* could be acquired by FAW during feeding on maize leaves, and to clarify the exact spatial distribution of this species within maize leaves and the digestive system of FAW, we fluorescently labeled *P. dispersa* strain with GFP (Fig. 2A). Three days after GFP-labeled *P. dispersa* was applied to maize roots or sprayed on the leaves, it was found to be located in roots (Fig. S2A-B) and in leaf veins (Fig. 2B, C). This indicated that environmental *P. dispersa* can successfully establish itself in maize roots and leaves. No fluorescence was observed in the maize leaves after the wild-type *P. dispersa* strain was inoculated into maize leaves (Fig. S2). Analysis of maize leaf sections revealed that *P. dispersa* was mainly concentrated in the sclerenchyma strands and vascular bundles (Fig. 2D, E). To further investigate whether *P. dispersa* was able to establish itself within the digestive system of FAW and, if so, to determine the exact site of colonization, we fed newly hatched aseptic FAW on maize leaves in which GFP-labeled *P. dispersa* had been previously inoculated onto maize roots. We found that *P. dispersa* exclusively colonized FAW Malpighian tubules, as indicated by the presence of GFP fluorescence (Fig. 2F–H, Fig. S3). To further reveal the exact presence of *P. dispersa* in FAW, we dissected FAW and then quantified the GFP abundance in different tissues using a qPCR approach. The result showed that *P. dispersa* mainly colonized the Malpighian tubules, followed by the gut, whereas fat body and cuticle almost showed no *P. dispersa* colonization (Fig. 2I). The reason why we did not observe fluorescence in the gut is probably due to the low abundance of *P. dispersa* in gut than that in Malpighian tubules ($2^{5.95}=62.0$ times, 2I), and the gut possessed much larger bulk than Malpighian tubules, resulting in dispersion of the GFP fluorescence. Moreover, *P. dispersa* showed comparative abundance in whole FAW caterpillar with that of Malpighian tubules (Fig. 2I).

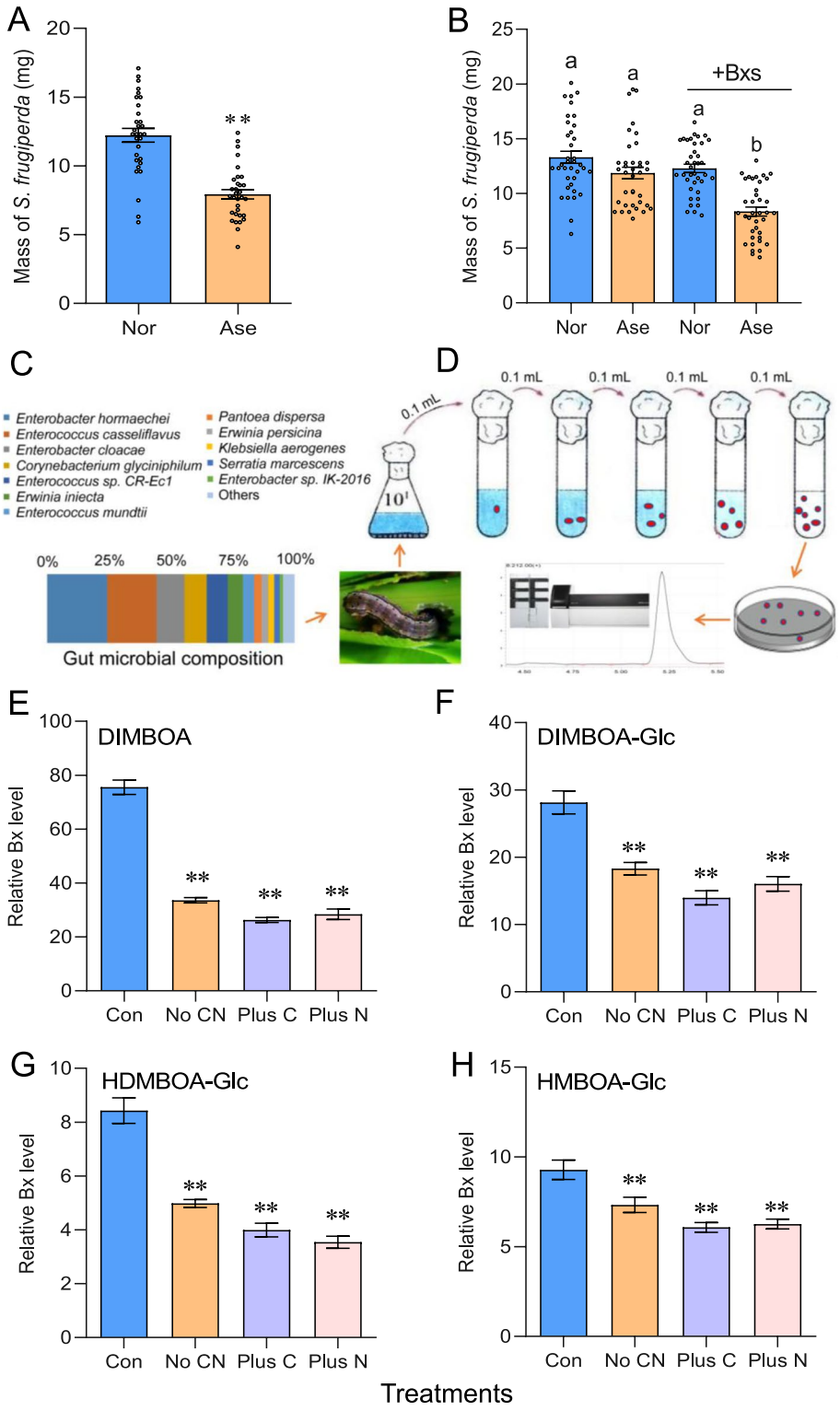
The Malpighian tubules are the main detoxification and metabolism organs in lepidopteran larvae and are actively involved in defenses against and metabolism of xenobiotic and toxic compounds [27]. Bxs determination results showed that levels of all the major Bx components were greater in the Malpighian tubules than in the midgut (Fig. 3A–E). This indicated FAW transports harmful Bx components to the Malpighian tubules to avoid subsequent poisoning.

P. dispersa assists larval growth by metabolizing Bxs

To determine whether colonization of the Malpighian tubules by *P. dispersa* is conducive to the growth of FAW, aseptic FAW larvae with and without *P. dispersa* inoculation were allowed to feed on wild-type and *bx2* mutant maize (in which Bxs were almost absent) [28]. Inoculation of aseptic FAW larvae with *P. dispersa* significantly increased FAW weight gain but showed no effect on the growth of FAW larvae when feeding on *bx2* mutants (Fig. 3F), demonstrating that *P. dispersa* affects the growth of FAW mainly through the metabolism of Bx compounds, rather than other metabolites.

(See figure on next page.)

Fig. 1 Isolation, cultivation, and identification of microorganisms metabolizing Bxs. **A** Mass of normal (Nor) and aseptic (Ase) *S. frugiperda* (FAW) larvae when feeding on surface-sterilized wild-type (WT) maize leaves and **B** when feeding on the artificial diet with or without Bxs. **C** The overall microbiome composition of normal FAW revealed by full-length 16S rRNA gene sequencing. **D** Guts from field-collected 5th instar larvae of FAW were ground in PBS buffer; microbial species were cultivated in medium with Bxs as the sole carbon and nitrogen source. After 7 rounds of enrichment, the obtained colonies were isolated on solidified media. The metabolic degradation of Bxs by individual colonies was revealed through LC–MS. The red dots symbolize the enriched species that can metabolize Bxs. **E**–**H** Quantitative determination of Bx levels following metabolism by *P. dispersa*. Bx levels were assessed under four treatments: with heat-inactivated *P. dispersa* inoculation (Con), with *P. dispersa* inoculation without other carbon or nitrogen (No CN), with *P. dispersa* inoculation with added glucose (Plus C), and with *P. dispersa* inoculation with added NH_4Cl (Plus N). The Bx levels were determined 3 h after inoculation at 28 °C. The data are means ± SEs. Asterisks indicate significant differences between the control and other treatments (Student's *t* test, $n=6$; **, $P<0.01$)



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To evaluate the overall contribution of *P. dispersa* to Bxs metabolism within the entire FAW larval gut microbiome, we performed both in vivo and in vitro experiments. For in vivo experiments, aseptic, normal (in the presence of the natural gut microbiome), and *P. dispersa*-inoculated FAW were starved for 4 h, followed

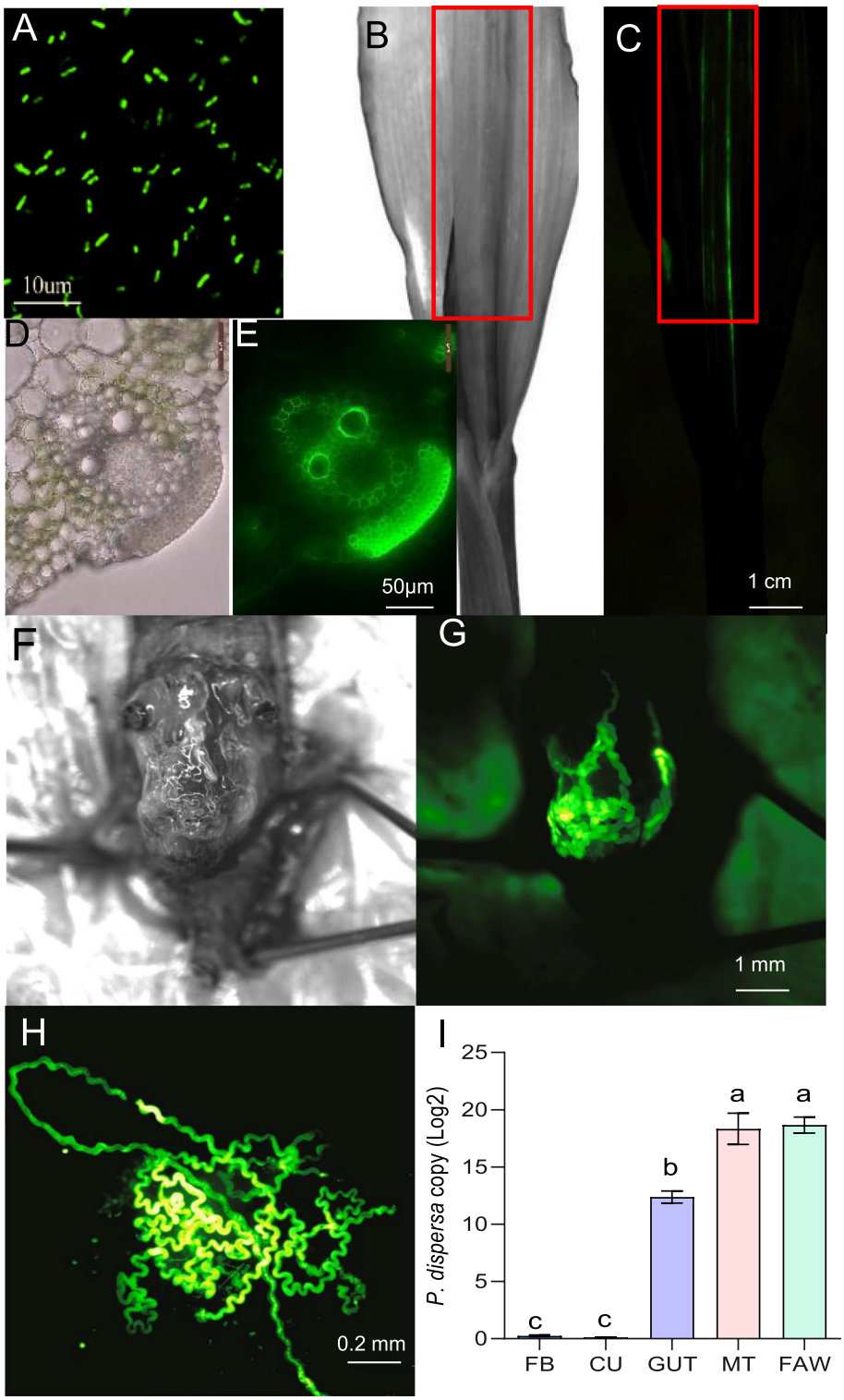

Fig. 2 Tissue-specific localization of *P. dispersa* in maize and FAW larvae. **A** Fluorescence micrographs of GFP-labeled *P. dispersa*. **B**, **C** Images showing maize leaves and **D**, **E** tissue slices after GFP-labeled *P. dispersa* inoculation into maize roots. **F**, **G** Images showing gut and Malpighian tubules of FAW after feeding on the aforementioned maize leaves. **H** The dissected Malpighian tubules. Plots **B**, **D**, and **F** were taken under visible light, and plots **C**, **E**, **G**, and **I** represent the same images under UV light. **I** Absolute abundance of *P. dispersa* in different tissues of FAW. FB, fat body; CU, cuticle; GUT, whole gut; MT, Malpighian tubules; FAW, whole FAW caterpillar. The data are means ± SEs. Different letters denote significant differences between treatments based on one-way ANOVA followed by Duncan's multiple comparison test at $P < 0.05$ ($n = 8$)

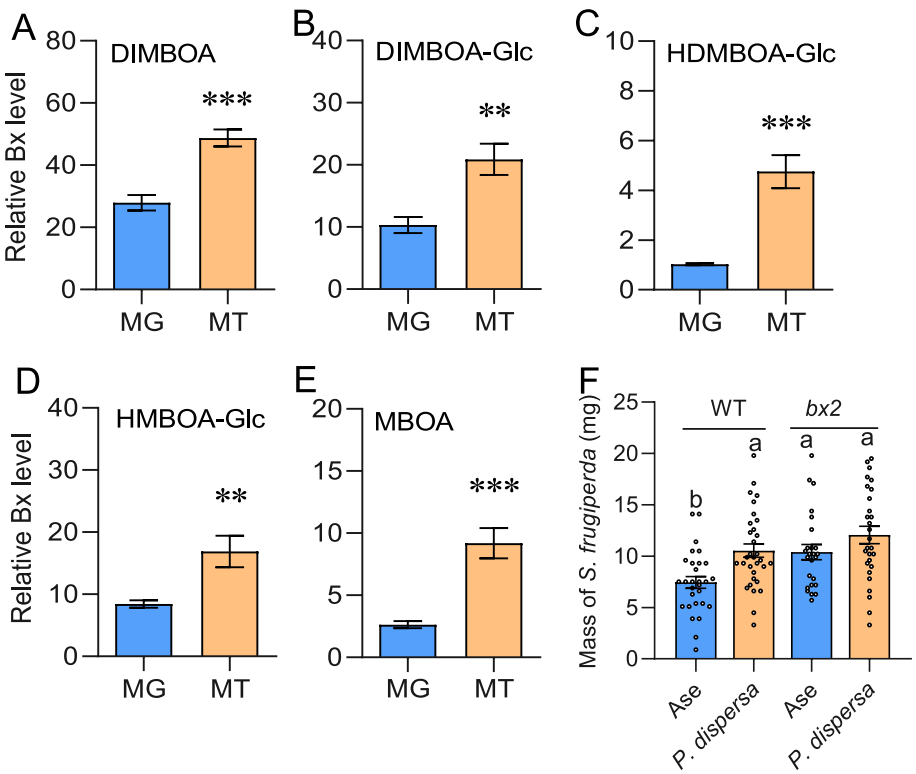

Fig. 3 Enrichment of Bxs in Malpighian tubules and role of *P. dispersa* in promoting FAW larval growth. **A–E** Aseptic FAW was fed an artificial diet until reaching the 5th instars, which were subsequently fed surface-sterilized maize leaves for additional 2 days. The midgut and Malpighian tubules were then dissected for Bxs determination after a 4-h starvation period. The data are means ± SEs. Asterisks indicate significant differences between tissues (**$P < 0.01$; ***$P < 0.001$; Student's *t* test, $n = 4$, every biological replicate represents a pool of 5 larval samples; MG: midgut, MT: Malpighian tubules). **F** Mass of aseptic (Ase) FAW larvae and *P. dispersa*-inoculated FAW larvae when feeding on WT and *bx2* mutants. Different letters denote significant differences between treatments based on one-way ANOVA followed by Duncan's multiple comparison test at $P < 0.05$ ($n = 25–37$)

by feeding on surface-sterilized maize leaves. Maize tissues in the gut of FAW was then immediately dissected for Bxs determination 3 h after feeding. We found that Bxs levels in guts from the normal and the *P. dispersa*-inoculated FAW groups were all significantly reduced compared with those in aseptic group (Fig. 4A–E). This demonstrates that both the entire FAW larval gut microbiome and the inoculated *P. dispersa* can reduce Bxs levels in the gut of FAW. For in vitro experiments, the gut contents from above mentioned treatments were first collected. Three hours after inoculation of these gut contents in crude Bxs extracts from maize leaves (containing Bxs and proteins, representing the actual nutritional status of FAW guts), the gut contents from both normal and *P. dispersa*-inoculated FAW groups significantly reduced Bxs levels compared to those in aseptic FAW group (Fig. S4A-E). Notably, the higher metabolism rate of HDMBOA-Glc and HMBOA-Glc by gut contents from naturally reared FAW larvae, as compared to those inoculated with *P. dispersa* (Fig. S4A-E), suggests the presence of other probiotics in the FAW gut that may contribute to Bxs detoxification. Moreover, the levels of Bxs in the frass of both normal and *P. dispersa*-inoculated group were significantly lower than those in the frass from the aseptic FAW (Fig. S4F), indicating that both natural gut microbiome and *P. dispersa*, as a key member in gut microbial community, assists FAW through detoxifying Bx compounds. What is more, by using Bxs as the sole carbon and nitrogen source, or using the crude extracts, the proliferation rate of *P. dispersa* (indicated by fold changes in microbial biomass, Fig. 4F) was 2.5 and 26.6, respectively. These data support that Bxs can be metabolized for the synthesis of other molecules to sustain the survival of *P. dispersa*, both with or without support from other nutrients.

Enrichment of *P. dispersa* by FAW

P. dispersa is a relatively common bacterium in the natural environment and a well-known maize endophyte with a nitrogen-fixing function, which can significantly promote the growth of maize, sugarcane, and other herbaceous plants [29]. Through the acetylene reduction ethylene reaction assay, we demonstrated the biological nitrogen fixation capability of *P. dispersa* (Fig. S5),

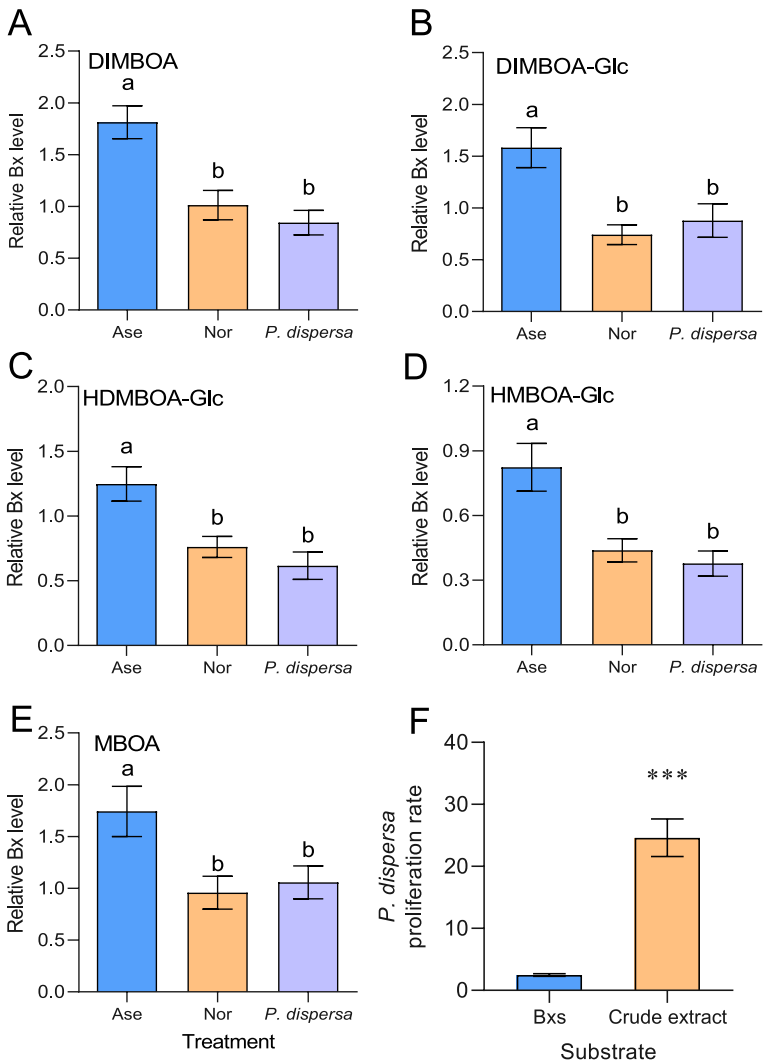

Fig. 4 Bxs metabolism by gut microbiome and *P. dispersa* in FAW. **A–E** The 5th instar of aseptic (Ase), normal (Nor, with the entire FAW larval gut microbiome), and *P. dispersa*-inoculated FAW were starved for 4 h, followed by feeding on surface-sterilized maize leaves for 3 h. Maize tissues in the gut of FAW was then immediately dissected for Bxs determination. Different letters denote significant differences between treatments based on one-way ANOVA followed by Duncan's multiple comparison test at $P < 0.05$ ($n = 5$). **F** The proliferation rate of *P. dispersa* under different substrates (Bxs and crude extract) was determined by centrifuging the cultures after 12 h of shaking at 28 °C and 200 rpm, measured as the fold changes in microbial biomass. The data are means ± SEs. Asterisks indicate significant differences between different substrates (Student's *t* test, $n = 6$; ***, $P < 0.001$)

confirming its pivotal role as a maize endophyte partly through facilitating nitrogen transformation and utilization by maize.

To examine the presence of this species in both FAW and maize in the natural environment, we investigated the absolute abundance of *P. dispersa* in maize and FAW in seven maize-growing farmlands in different geographic regions in China using qPCR. We found that the presence of *P. dispersa* in FAW guts (including the Malpighian tubules) and maize leaves was ubiquitous, and moreover, its absolute abundance in FAW guts was significantly greater than that in maize leaves collected from the same fields (Fig. 5A), indicating that FAW can significantly enrich *P. dispersa* in the gut through feeding on maize leaves. Furthermore, we found that the absolute abundance of *P. dispersa* in 2-month-old wild-type maize leaves was also significantly greater than that in *bx2* mutant leaves (Fig. 5B), implying that the Bxs secreted by maize roots may be essential for recruiting and the growth of *P. dispersa*. Taken together, these field datasets demonstrate that *P. dispersa* widely colonizes both FAW and maize in natural farmlands. Moreover,

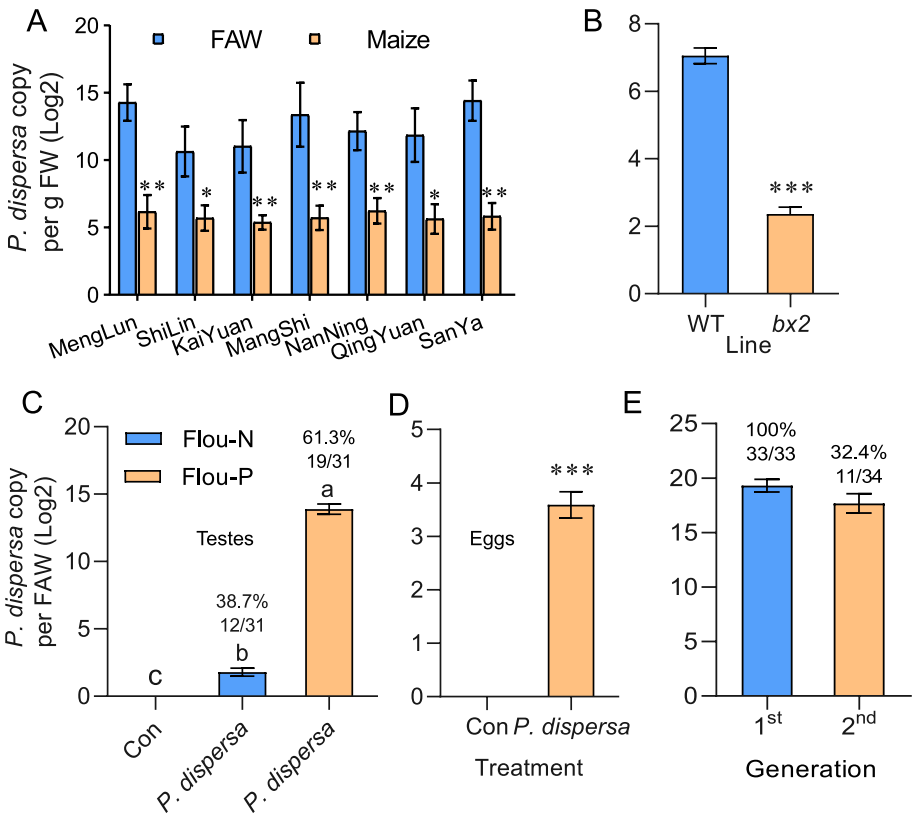

Fig. 5 *P. dispersa* detected in maize leaves and FAW and its vertical transmission. **A** Absolute abundance of *P. dispersa* in the gut of field-collected FAW and in the FAW-infested maize leaves, detected in pairs from seven regions across Southern China. FW: fresh weight. **B** Absolute abundance of *P. dispersa* detected in the leaves of two-month-old wild-type (WT) maize and *bx2* mutants. **C–E** Absolute abundance of *P. dispersa* detected in the testes (**C**), eggs (**D**) (every 10 eggs, laid out by GFP-labeled *P. dispersa* inoculated FAW), and Malpighian tubules (**E**) (1st and 2.nd represent the first and the second generation, respectively). Con, control; Folu-N, fluorescence-negative; Folu-P, fluorescence-positive. The data are means ± SEs. Asterisks indicate significant differences between treatments (Student's *t* test, $n = 6–8$; $^{**}P < 0.01$; $^{***}P < 0.001$)

the enrichment of *P. dispersa* in maize as an endophyte appears to be associated with Bx compounds.

Vertical transmission of *P. dispersa* in FAW

The vertical transmission of commensal bacteria has been shown to play an important role in the growth and development of insects [13, 14]. To assess whether the transformation of *P. dispersa* from a maize endophyte into a probiotic for FAW is vertically transmissible to subsequent generations, we dissected pre-pupal stage FAW inoculated with GFP-labeled *P. dispersa* and observed GFP fluorescence in their testes (Fig. 5C, Fig. S6A-B). Notably, 19 out of 31 male pre-pupal stage's FAW testes exhibited GFP fluorescence, with *P. dispersa* abundance significantly higher than that showed no GFP fluorescence (indicated by copy number, $2^{13.9}$ vs. $2^{1.8}$, Fig. 5C). Similarly, we observed GFP fluorescence in some FAW eggs following GFP-labeled *P. dispersa* inoculation (Fig. S6C-D). DNA extraction from these eggs, followed by qPCR, revealed that pre-inoculation of FAW larvae with *P. dispersa* significantly increased its presence in the eggs (Fig. 5D). Furthermore, after GFP-labeled *P. dispersa* inoculation, GFP fluorescence was observed in 100% (33/33) of the first-generation and 32.4% (11/34) of the second-generation FAW in their Malpighian tubules. Moreover, the abundance of *P. dispersa* in the Malpighian tubules that showed fluorescence was similar between the two generations (Fig. 5E). Those data suggest that after conversion to a probiotic of FAW, *P. dispersa* strains can be vertically spread through FAW eggs for intergenerational transmission. However, it should be note that although *P. dispersa* established in all first-generation FAW larvae's Malpighian tubules, only 61.3% of FAW showed further establishment by *P. dispersa* in their testes (Fig. 5C–E).

P. dispersa does not promote the growth of *M. separata*

To explore whether this herbivore–microbe relationship is unique to FAW, we examined the role of *P. dispersa* in supporting the growth of another maize pest,

M. separata, which shares a common lineage with FAW within the Noctuidae family. Herbivore performance was tested using aseptic *M. separata* with or without *P. dispersa* inoculation. The inoculation of *P. dispersa* had no obvious effect on the growth of *M. separata* when feeding on maize leaves (Fig. S7), implying that *P. dispersa* did not settle in the digestive system of *M. separata* or that the habitat within the digestive system of *M. separata* larvae was not favorable for the metabolism of Bxs by *P. dispersa* after colonization. This also suggested that the microenvironment in the guts of lepidopteran larvae of different species may have an impact on the colonization or function of certain microbial species.

Different herbivores enrich distinct gut microbiomes

We then sequenced the gut microbes from both FAW and two frequently reported maize herbivores (*M. separata* and *Spodoptera litura*) after aseptic larvae had been allowed to feed on the same greenhouse-grown maize leaves until the 5th instar. We found that the community assemblages of the gut microbiota were distinctly different among the three insect species (Fig. 6A). Principal coordinate analysis revealed that the initial two axes explained 56.1% of the total variation in gut bacterial communities among these insects. The first axis (explaining 33.7% of the variance) distinctly differentiated FAW and *S. litura* from *M. separata*, whereas the second axis (explaining 22.4% of the variance) separated FAW from *S. litura* by their gut microbiota assemblages. The gut bacterial assemblages differed significantly ($p=0.005$; PERMANOVA) among all three insects (Fig. 6A).

The taxa with the highest abundance in the guts of the three insect species were Proteobacteria, followed by Actinobacteria and Firmicutes. Notably, *P. dispersa* exhibited a significantly greater abundance in the FAW than in the other two insect species. Its relative abundance within the gut microbiota of FAW ranged from 1.44% to 15.44% of all commensal species. From an evolutionary perspective, *P. dispersa* is phylogenetically related to *Erwinia persicina* and *Erwinia iniecta*, both of which belong to the

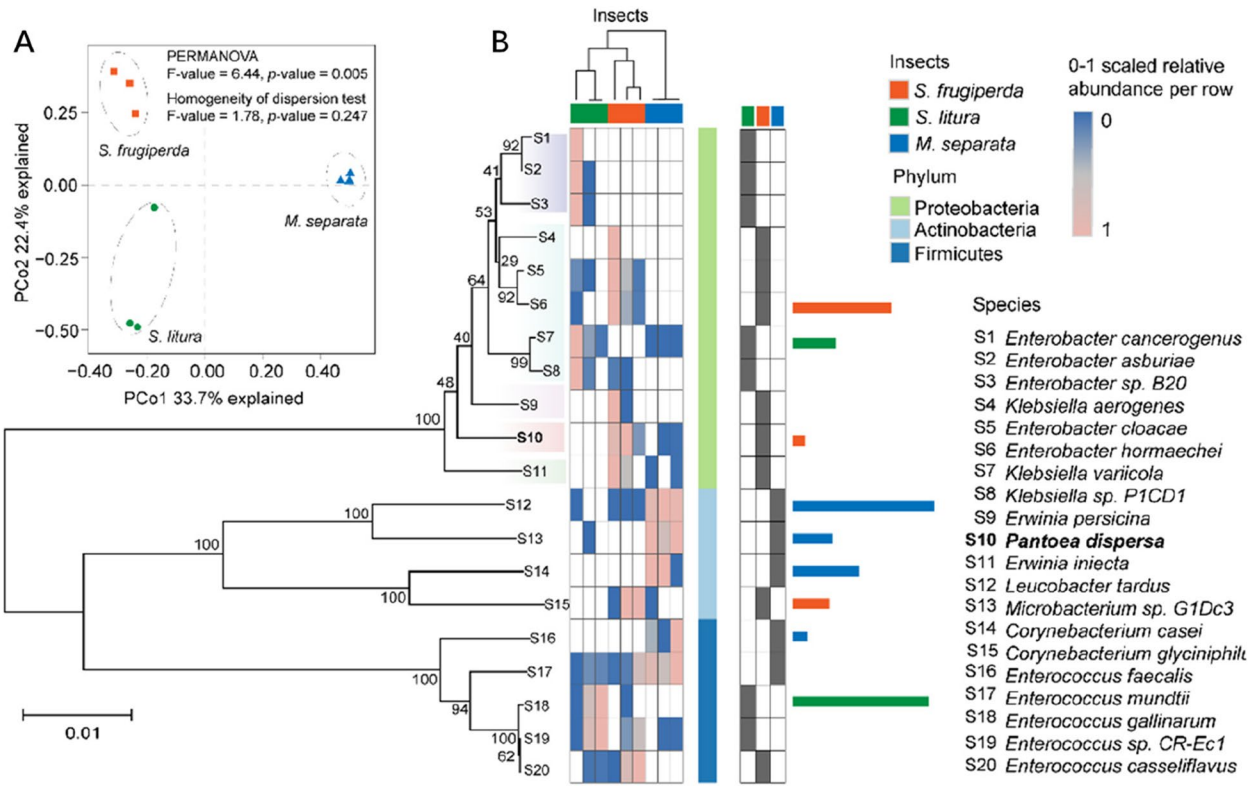

Fig. 6 Gut microbiomes of three insects determined via full-length bacterial 16S rRNA gene sequencing. **A** Gut bacterial β diversity between three insects illustrated using principal coordinate analysis, followed by permutational analysis of variance and a homogeneity of dispersion test. **B** Phylogenetic tree plot of the top 20 ASVs and a heatmap plot with the hierarchical clustering of all samples showing the relative abundance (%) of each ASV among the three insects. Here, a relative color scheme was employed by covering the minimum and maximum values in each row (i.e., each ASV) with colors. Differences in the relative abundance of each ASV among the three insects were further examined using the nonparametric Wilcoxon's *U* test. For ASVs that were significantly enriched, the highest abundances in the corresponding insect gut are shown in bar charts on the right side of the heatmap plot

phylum Proteobacteria. Interestingly, all the ASVs that demonstrated greater abundance in *M. separata* than in the other two insects were Actinobacteria, whereas those exhibiting greater abundance in FAW and *S. litura* were both Proteobacteria and Firmicutes (Fig. 6B). Overall, the gut microenvironments of the larvae of different species of lepidopterans could shape their unique gut microbial signatures, with *P. dispersa* being particularly enriched in the gut of FAW when feeding on maize leaves.

Exploration of the candidate genes involved in metabolizing Bxs in *P. dispersa*

To explore the pivotal genes within *P. dispersa* that are responsible for Bxs metabolism, we performed genomic sequencing of *P. dispersa* and transcriptomic sequencing before and after the introduction of Bxs. Genome sequencing revealed that the *P. dispersa* genome has a chromosome size of 4.15 Mb and a GC content of 57.70%, encompassing a total of 3824 encoded genes. The *P. dispersa* genome contained two plasmids: plasmid 1, 677 kb in length with a GC content of 57.67%, which encoded 646 genes, and plasmid 2, 157 kb in length with a GC content of 53.88%, which encoded 188 genes. The total genome size was 4.99 Mb (Fig. S8A-C).

A total of 396 genes were upregulated, and 237 genes were downregulated upon the introduction of Bxs compared with those in the controls (Table S2). Of the top 10 highly expressed genes, 7 originated from plasmids. The expression of two gene clusters was strongly induced in plasmids. Cluster one included seven genes (plasmid2_41-47, named No.#1-#7), five of which were among the top 10 most strongly upregulated genes. Cluster two included two genes (plasmid1_61-62, named No. #8-#9), both of which were among the top 10 most strongly upregulated gene (Fig. S8D, Table S2). These findings imply that genes in plasmids of *P. dispersa* may play a crucial role in metabolizing Bxs as the sole carbon and nitrogen source.

The eggNOG functional annotation revealed that genes in the plasmids upregulated in response to Bxs metabolism were mainly involved in energy production and conversion and carbohydrate transport (Table S2). GO enrichment suggested that the organic acid biosynthetic process, carboxylic acid biosynthetic process, and cellular amino acid metabolic process were involved in the metabolism of Bxs (Fig. S9). The most enriched GO processes were the organic nitrogen compound metabolic process ($p=0.001$) and the organic substance metabolic process ($p=0.03$) (Fig. S10). Surprisingly, 34 genes involved in flagellin biosynthesis or movement were upregulated (Table S3), implying that Bxs may serve as important signals to attract *P. dispersa* by promoting flagellin biosynthesis or movement. However, this needs further investigation.

To investigate which *P. dispersa* genes are involved in the metabolism of Bxs, we successfully cloned 9 genes (i.e., abovementioned #1–#9 in two gene clusters) that were highly expressed during the process of Bxs metabolism. After they were inserted into the pet32a + vector and introduced into the BL21 strain for protein expression, six of these genes were fully translated into proteins (Fig. 7A). We performed enzyme activity assay using the purified proteins, only proteins translated from the target genes #1 and #7 (i.e., plasmid2_41 and plasmid2_47, annotated as pre-guanitoxin N-hydroxylase GntA and MOSC domain-containing protein, respectively) were involved in Bxs metabolism (Fig. S11).

To confirm the above findings, we performed Bxs metabolism assays using cell strains that expressed target genes #1 and #7 and obtained similar results (Fig. 7B–E), further strengthened that the proteins encoded by target genes #1 and #7 are involved in Bxs metabolism. Additionally, #1 and #7 played distinct roles in the metabolism of different Bx compounds. The ability of protein #1 metabolizing DIMBOA and DIMBOA-Glc was greater than that of protein #7, but there was no difference in the ability of metabolizing HDMBOA-Glc between them. To support this finding, we obtained another *P. dispersa* strain (hereafter referred to as OsPD), which was previously identified as a core member of the microbiota in rice and can be vertically transmitted as a seed endophyte [30, 31]. Gene-specific primer cloning and sequencing revealed that OsPD did not produce PCR products for candidate genes #1–#7 but did harbor candidate genes #8 and #9, although those two genes exhibited some SNPs compared to those in our screened *P. dispersa* strain (hereafter referred to as ZmPD) (Fig. S12-S15). Compared with OsPD, ZmPD presented higher Bxs metabolism rates (Fig. 7B–E), which verified the role of target genes #1 and #7 in ZmPD strains in metabolism Bxs. Therefore, different strains of *P. dispersa* may possess diverse genes in addition to the aforementioned genes #1 and #7 that are involved in Bxs metabolism.

Discussion

The global spread of FAW poses a significant threat to maize production, and this widespread threat appears to be intricately tied to its gut microbiota. Bxs, especially HDMBOA-Glc, are effective anti-FAW compounds produced by maize to defend FAW attack [7]. As nitrogen-containing organic compounds, Bxs can serve as nitrogen and carbon source for a diverse array of environmental bacteria, including those inhabiting the guts of maize-feeding insects. After 7 rounds of cultivation, agar plate planting, and Bxs metabolism analyses using LC–MS, we

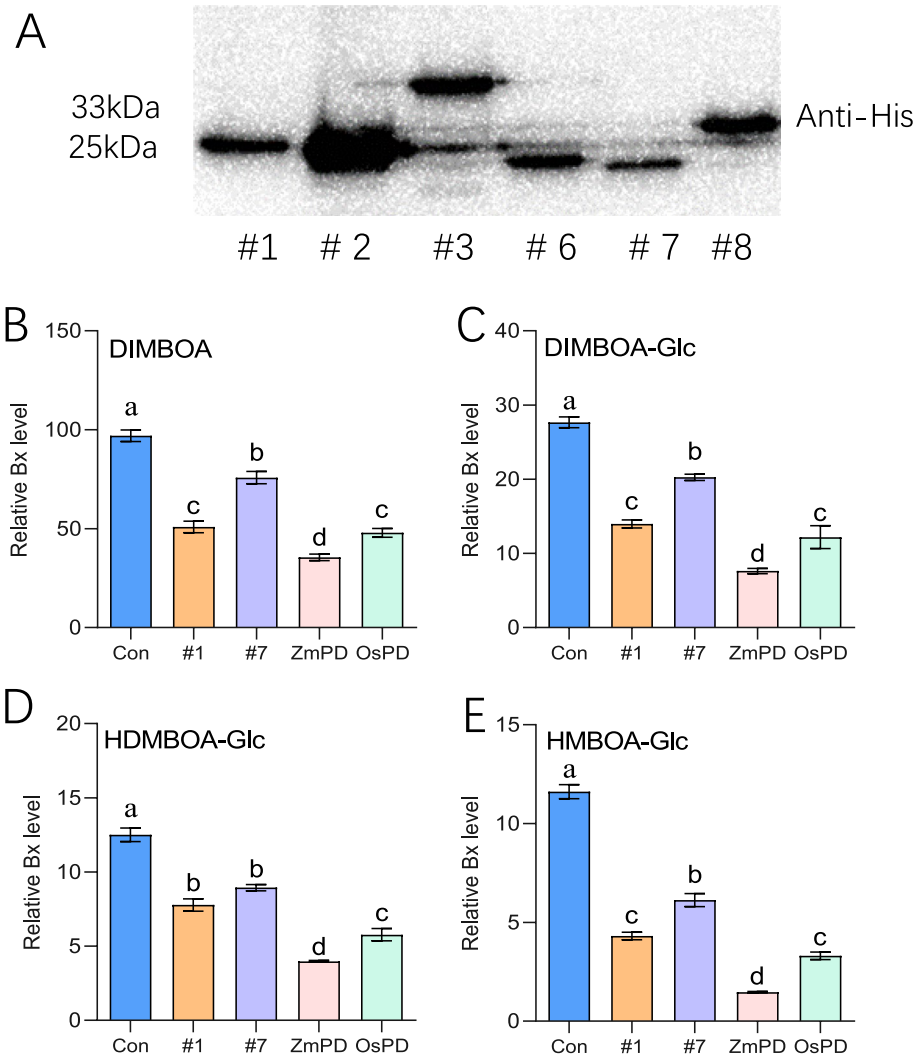

Fig. 7 Identification of candidate genes involved in Bxs metabolism. **A** Western blot of the purified proteins from candidate gene expression products. **B–E** Quantitative determination of Bx levels following metabolism in BL21 cells expressing candidate genes (#1 and #7), with BL21 cells expressing pet32a + empty vector serving as a control (Con). ZmPD represents *P. dispersa* strains screened in this study and OsPD represents strains previously found in rice. Determination of Bxs was performed 3 h after cell inoculation at 28 °C. The data are means ± SEs. Different letters denote significant differences between treatments based on one-way ANOVA followed by Duncan's multiple comparison test at $P < 0.05$ ($n = 6$)

successfully obtained *P. dispersa* from the gut of FAW. Upon inoculation of maize roots with GFP-labeled *P. dispersa*, we observed bacteria in the vascular system of both maize leaves and roots, which is similar to the findings for another *P. dispersa* strain found in sweet potato (*Ipomoea batatas*), where root inoculated *P. dispersa* was further observed in the leaf petiole [32]. When aseptic FAW fed these maize leaves, *P. dispersa* was able to establish itself in the Malpighian tubules and guts of FAW larvae. Herbivore performance bioassays revealed that *P. dispersa* significantly promoted FAW growth, and this promotion effect was directly associated with the metabolism of Bxs as revealed via bioassays performed on both artificial diet and maize seedings (*bx2* mutants) with or without Bxs (Figs. 1 and 5). *P. dispersa* can further establish itself in the testes at the prepupal stage. Moreover, we observed fluorescence in eggs from the first generation and Malpighian tubules from the second generation, and the abundance of *P. dispersa* was also confirmed (Fig. 5C–E, Fig. S6). It indicates that *P. dispersa* can be vertically spread through eggs for intergenerational transmission. Similarly, the bacterium *Asaia* has been found in the spermatic bundles and Malpighian tubules of the host insect (*Scaphoideus titanus*), which can also be vertically transmitted [33]. The simultaneous localization in both maize and FAW, as well as the conversion of

maize endophytes into FAW probiotics, is demonstrated for the first time in this study. Our field sampling further confirmed the coexistence of *P. dispersa* in FAW guts and maize leaves collected from seven independent natural farmlands. The pronounced enrichment of this species in the FAW gut underscores its role as a crucial member of the gut microbial community, potentially contributing to the widespread distribution of FAW.

P. dispersa is a common environmental microbe. Previous research has focused primarily on its ability to fix nitrogen [12]. It has been demonstrated to promote the growth of many economic crops, including maize, sugarcane, wheat, and rice [17, 29]. We also confirmed the nitrogen-fixing ability of *P. dispersa* as a maize endophyte (Fig. S5). Moreover, as a member of the insect gut microbiome, *P. dispersa* has been found to confer benefits to diverse insect orders. For instance, female *Bactrocera dorsalis* fed a diet supplemented with *P. dispersa* lay more eggs than controls [34]. *P. dispersa* reduces the mortality rate and is essential for the normal growth of the stinkbug *Plautia splendens* [13], highlighting the mutual relationship between *P. dispersa* and certain insects. In parallel, in our study, we present a first study showing that *P. dispersa* can also effectively improved the fitness of FAW as a probiotic, by facilitating the detoxification of Bxs. This occurred during insect feeding, as the maize-colonized *P. dispersa* was recruited and further proliferated within the FAW Malpighian tubules and the guts. Other studies have shown that certain *Pseudomonas* species colonizing the Malpighian tubules play a role in the transstadial passage from larvae to adult mosquitoes, specifically in *Anopheles stephensi*, thereby contributing to mosquito paratransgenesis [35]. The bacteria found in the Malpighian tubules likely originate from the gut, as these tubules are directly connected to it. On one hand, the Malpighian tubules serve as detoxification organs for lepidopteran larvae, where they accumulate toxic compounds originated from host plants. On the other hand, these compounds can also be metabolized by probiotics colonized in these organs [27].

In our study, the *P. dispersa* isolated from the gut of FAW was likely recruited from the soil environment due to the exudation of Bxs from maize roots. The primary and secondary root metabolites are key regulators that shape rhizobiome microbial assemblages [36]. Another possibility is that *P. dispersa* just attached to maize leaves without becoming an endophyte and can be consumed by FAW, as previously reported [37]. Most of all, Bxs exuded from roots or apoplasts is a key trait in maize for the recruitment of certain microbes [38, 39]. *P. dispersa* may migrate and settle through root absorption processes as indicated by GFP fluorescence in the vascular system (Fig. S2, Fig. 2B–E). Furthermore, we found *P. dispersa* can utilize Bxs over a wide range of pH values (Fig. S16), which indicated the strong ability of *P. dispersa* to adapt Bxs metabolism.

Previous reports have also indicated the strong ability of FAW to harness gut microbes. For instance, when faced with nutrient-deficient food, nutrient utilization efficiency is enhanced in FAW larvae through the aid of gut microbes [40]. In addition, *Klebsiella* sp. isolated from the gut of FAW can decompose PVC plastic as an energy source in the absence of other foods [41]. Moreover, *Pseudomonas* spp. isolated from field-collected FAW larvae can utilize pesticides as their sole carbon source to enhance FAW resistance to pesticides [42]. These lines of evidence help to explain the widespread distribution of FAW. Our study provides additional insights into the role of *P. dispersa* as a crucial gut microbe that assists FAW feeding on maize leaves and illustrates the triangular relationships among herbivores, microbes, and plants. Indeed, a cohort of microorganisms may contribute to FAW fitness. However, our study cannot rule out the possibility that other unidentified bacterial species act as probiotics in the FAW gut by participating in the detoxification of Bxs. For example, certain candidate bacteria may exhibit strong Bxs degradation activity only with the support of additional nutrients, and there may also be synergistic interactions among different bacterial species that can enhance this detoxification process. This necessitates further investigation.

In natural environments, leaf microbiomes can be enriched in the guts of herbivorous insects following feeding [37], which heavily relies on insect identity, adaptability, and fitness to certain host plants. We found a remarkable prevalence of *P. dispersa* in both the FAW gut and maize leaves in natural farmlands, whereas the inoculation of *P. dispersa* had no obvious effect on the growth of *M. separata*. Moreover, the relative abundance of *P. dispersa* in the gut of FAW was significantly greater than that in the guts of *M. separata* or *S. litura* fed the same greenhouse-cultured maize leaves (Fig. 6B). These findings suggested that the microbial colonization patterns are specific to certain insect species and underscore the importance of subtle differences in the gut microenvironment in facilitating such colonization [40].

Pantoea spp. have been observed in association with many other hosts, including soybean, birds, fish, ruminants, and humans [35]. However, the ability of these bacteria to colonize hosts has remained unpredictable, and the host range and colonization patterns for most of the isolates are largely unknown. *Pantoea* spp. accounted for the largest proportion of all isolated caffeine-degrading bacteria from *H. hampei* guts, highlighting their crucial role in detoxifying special chemicals (e.g., secondary metabolites from host

plants) detoxification [15]. The settlement of *P. dispersa* in the gut of FAW, as opposed to other lepidopteran species, suggested a high degree of compatibility between *P. dispersa* and FAW. The plasmid in *Pantoea* spp. was originally derived from an ancestral plasmid that encodes a larger array of proteins play roles in the adaptation to various ecological niches [35]. As evidence, genes located on the plasmid were more upregulated than those on the chromosomes when responding to Bxs metabolism (Fig. S8D, Table S2). Moreover, the protein encoded by target gene #1 was involved in nitrogen assimilation. Given the long-term evolutionary history of *Pantoea* spp., diverse proteins in different strains (such as ZmPD and OsPD) might be involved in the metabolism of Bxs (Fig. 7B–E).

Symbiotic microorganisms serve as vital driving forces for the long-term evolution of insects and plants. During symbiosis, microbial genes can integrate into the host, enabling the host to acquire new functions. The most classic example involves chloroplasts and mitochondria, which have long been assumed to be derived from exogenous microorganisms gradually transition into plant organelles [43]. In this study, the maize endophyte *P. dispersa* was converted to a probiotic in the FAW gut, which was subsequently transferred to the next generation through eggs, suggesting evolutionary interactions between *P. dispersa* and FAW. We provided hints to support the occurrence of a coevolutionary event between *P. dispersa* and FAW through the transfer of genes, which can endow FAW with its own detoxification capabilities, potentially reducing its vulnerability to maize chemical defense during feeding. However, further investigation into this topic is warranted.

Conclusions

In summary, our study revealed that FAW can effectively enrich and recruit *P. dispersa*, a prevalent endophyte in maize, as a member of gut microbiota through feeding. This transformation effectively converts *P. dispersa* into a beneficial probiotic for FAW, by metabolizing anti-herbivore Bx compounds and fostering the growth of FAW. Moreover, the acquired *P. dispersa* can be passed on to the next generation through vertical transfer. The gene clusters within the plasmids of *P. dispersa* involved in Bxs metabolism. These findings offer evidence for the rapid global spread of FAW and provide insights into the complex interactions among plants, pests, and microorganisms. Here, we unveil a remarkable strategy employed by FAW, in which it transforms its host's allies into its own allies, which then aids its rapid adaptation to the antiherbivore compounds accumulated by maize and ultimately facilitates its extensive worldwide distribution.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-024-01957-z.

Additional file 1: Fig. S1. Map of the geographic locations of the field-collected samples. The latitudes and longitudes of the seven collection sites are as follows: MengLun: 21.892319 N, 101.280868 E; KaiYuan: 23.66561 N, 103.280817 E; ShiLin: 24.41086 N, 98.495312 E; MangShi: 24.41086 N, 98.495312 E; QingYuan: 24.633446 N, 112.331656 E; NanNing: 22.64309 N, 108.234571 E; and SanYa: 18.386363 N, 109.143506 E. Fig. S2. Fluorescence micrographs of maize roots (A-C) and leaves (D-E) taken under visible light (C-D) and UV light (A, B, E). The exposure conditions are the same as those in Fig. 2. Fig. S3. Bright field of Malpighian tubules dissected from FAWlarvae. Fig. S4. Bxs detected in maize crude extract and frass of FAW after metabolism by gut microbiome and *P. dispersa*.(A-E) The 5th instar of normal (Nor, with entire FAW larval microbiome), aseptic (Ase), and *P. dispersa*-inoculated FAW were starved for 4 h. Gut contents from each FAW were collected and inoculated with 200 µl maize crude extract for 3 h, and (F) frass was collected after FAW feeding on surface-sterilized maize leaves for 24 h. In frass, the levels of HDMBOA-Glc were below limits of detection. Different letters denote significant differences between groups based on one-way ANOVA followed by Duncan's multiple comparison test at $P < 0.05$ ($n = 5$). Fig. S5. Nitrogen fixation by *P. dispersa*. Nitrogen fixation activity measured by acetylene reduction. The data are means ± SEs. Asterisks indicate significant differences between the control (Con, *P. dispersa* cultured in media supplemented with NH_4Cl) and treatment (*P. dispersa* cultured in media not supplemented with NH_4Cl) groups (Student's *t* test, $n = 6$, ***, $P < 0.001$). Fig. S6. Fluorescence micrographs of FAW testes (A-B), eggs (C-D), and the next generation's Malpighian tubules taken under visible light (A, C, E) and UV light (B, D, F, G). The exposure conditions are the same as those shown in Fig. 2. Fluorescence in eggs is marked by the red arrows. Fig. S7. No effect of *P. dispersa* on the larval growth of *M. separata*. Aseptic *M. separata* without (Con) or with *P. dispersa*inoculation were fed on wild-type (WT) maize leaves for a period of 10 days. The data are presented as the means ± SEs; n= 30. Fig. S8. Candidate genes involved in Bxs metabolism revealed by genome and transcriptome sequencing of *P. dispersa*. (A) Gene map of the *P. dispersa* chromosome and (B-C) gene map of the two *P. dispersa* plasmids. (D) Top upregulated genes related to Bxs metabolism with a log2-fold change > 4 between the control (Con1-3) and Bxs treatment (BX1-3). Fig. S9. GO enrichment analysis of *P. dispersa* genes whose expression was altered in response to Bxs as the sole carbon and nitrogen source. Fig. S10. GO metabolic processes of *P. dispersa* genes when Bxs served as the sole carbon and nitrogen source. Fig. S11. Identification of purified proteins translated from candidate genes involved in Bxs metabolism. Quantitative determination of Bxlevels following metabolism by the purified proteins translated from candidate genes, with protein from empty vector as a control (Con).Determination of Bxs was performed 3 h after inoculation at 28°C in Hepes buffer (pH 7.0). The data are means ± SEs. Different letters denote significant differences between groups based on one-way ANOVA followed by Duncan's multiple comparison test at $P < 0.05$ ($n = 5$). Fig. S12. Nucleotide sequence alignment of the target genes in two *P. dispersa* strains (OsPD and ZmPD). Number 61 indicates the target gene No. #8. Fig. S13. Nucleotide sequence alignment of the target genes in two *P. dispersa* strains (OsPD and ZmPD). Number 62 indicates the target gene No. #9. Fig. S14. Amino acid sequence alignment of the target genes in two *P. dispersa* strains (OsPD and ZmPD). Number 61 indicates the target gene No. #8. Fig. S15. Amino acid sequence alignment of the target genes in two *P. dispersa* strains (OsPD and ZmPD). Number 62 indicates the target gene No. #9. Fig. S16. Metabolism of Bxs by *P. dispersa* under different pH conditions. The levels of Bxs metabolized by *P. dispersa* at 28°C after a 3-hour incubation at pH 5-10 (as the numbers indicated in the abscissa axis). Data are means ± SEs. Asterisks indicate significant differences between control (Con) and each treatment (e.g., 5+PD indicates *P. dispersa* incubated under a pH of 5) (Student's *t* test, $n = 6$-8; *, $P < 0.05$;**, $P < 0.01$; **, $P < 0.001$).

Additional file 2: Table S1. The target gene IDs and primers used in this study. Table S2. All differentially expressed genes in response to Bxs serving as the sole carbon and nitrogen source. Table S3. All differentially

expressed genes involved in flagellin biosynthesis or movement in response to Bxs serving as the sole carbon and nitrogen source.

Additional file 3: Supplementary note 1 Detailed information on the materials and methods.

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Authors' contributions
HW and JQ designed the project. JQ, FX, XL, JL, HW, SL, HY, YX and HW performed the experiments or data analysis. JQ wrote the manuscript, and the other coauthors contributed by commenting on and revising it. All the authors read and approved the final manuscript.

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Data availability
All the raw sequence data have been deposited in the NCBI Sequence Read Archive under the accession numbers PRJNA954674, PRJNA998569, and PRJNA1002094. You can find them with the following links: https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA954674; https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA998569; https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1002094.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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