Fall armyworm-associated gut bacteria modulate plant defense responses

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Mechanical damage caused by insect feeding along with components present in their saliva and oral secretions are known to induce JA-mediated defense responses in plants. This study investigated the effects of bacteria from oral secretions of the fall armyworm (FAW), *Spodoptera frugiperda*, on herbivore induced defenses in tomato and maize plants. Using culture-dependent methods, we identified seven different bacterial isolates, belonging to the family Enterobacteriacea, from the oral secretions of field-collected caterpillars. Two isolates, *Pantoea ananatis* and Enterobacteriaceae-1, down regulated the activity of the plant defensive proteins polyphenol oxidase (PPO) and trypsin proteinase inhibitors (trypsin PI), but up regulated peroxidase (POX) activity in tomato. *Raoultella* sp. and *Klebsiella* sp. down regulated POX but up regulated trypsin PI in this plant species. Conversely, all of these bacterial isolates up regulated the expression of the herbivore-induced maize proteinase inhibitor (mpi) gene in maize. Plant treatment with *P. ananatis* and Enterobacteriaceae-1 enhanced caterpillar growth on tomato, but diminished their growth on maize plants. Our results highlight the importance of herbivore-associated microbes and their ability to mediate insect plant interactions differently in host plants fed on by the same herbivore.

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INTRODUCTION

Physical damage caused by feeding of chewing insects induces jasmonic acid (JA)-mediated defense responses in plants. The magnitude of these responses is often modified by insect-derived cues present in their saliva, oral secretions (OS) and frass (Acevedo et al. 2015; Ray et al. 2015). The saliva of lepidopteran larvae is mainly produced by the labial salivary glands and released extra-orally through the spinneret (Felton et al. 2014). Some proteins present in caterpillar saliva can directly interact with plants to enhance or suppress herbivore-induced defenses. Previous studies have shown that glucose oxidase (GOX) (Musser et al. 2002; Tian et al. 2012), adenosine triphosphatases (ATPases) (Wu et al. 2012) and a recently identified phospholipase C (PLC) (Peiffer et al. unpublished) modulate JA-mediated defense responses in several host plants. Different from saliva, oral secretions or regurgitant arise from an insects foregut (Grant 2006) and contain fatty acid amino acid conjugates (FACs), insect and plant-derived enzymes, and microbes that modulate defense responses in plants (Chung et al. 2013; Schmelz 2015). Insect frass contains plant proteins, insect-derived proteins and microbes that regulate plant defenses (Ray et al. 2016). A large volume of work has been dedicated to the identification of elicitors and effectors (molecules that modulate plant defenses) present in insect saliva and oral secretions but there is very little known about the effects of herbivore-associated microbes on regulation of plant defenses.

Insects harbor diverse microbial communities that influence their interaction with other trophic levels and their environment. Some known services provided by insect-associated microorganisms include nutrient provisioning, synthesis of pheromone components, regulation of insect-immune responses and protection against parasites (Engel and Moran 2013; Douglas 2015). Microbes are essential for plant-feeding insects whose diet is generally low in nutrients and/or high in chemical defenses. Symbionts associated with phytophagous insects provide essential amino acids (Douglas 2015), aid in digestion (Vistto et al. 2009) and detoxify plant secondary metabolites including terpenes and phenolics (Hammer and Bowers 2015). For instance the phytophagous gypsy moth, *Lymantria dispar*, harbors symbiotic bacteria of the genus *Acinetobacter* that appear to metabolize toxic phenolic glycosides from its host plant *Populus tremuloides* (Mason et al. 2016). In other cases, association with specific microbes influences the insects' ability to utilize specific host plants; for example, fecundity of the pea aphid, *Acyrthosiphon pisum*, on white clover is highly dependent on the presence of the endosymbiont pea aphid U-type (Tsuchida et al. 2004). Similarly, the stinkbug *Megacopta punctatissima* owes its ability to use legume crops to the associated symbiont *Ishikawaella capsulate* (Hosokawa et al. 2007). Therefore, the capability of some phytophagous insects to exploit particular host plants seems to be, at least in part, mediated by their association with specific microorganisms.

Besides aiding in nutrition and detoxification, microorganisms associated with plant-feeding insects can also regulate induced plant defenses. This regulation can be indirect by affecting herbivore physiology or behavior, which can, in turn, modify their perception by plants (Zhu et al. 2014). Alternatively, microbes present in insect oral secretions come into direct contact with plant wounds during insect feeding; upon recognition of microbe-derived molecules, plants activate defensive pathways that can further affect the fitness of their herbivore host (Zhu et al. 2014). For example, the Colorado potato beetle, *Leptinotarsa decemlineata*, harbors gut endosymbiotic bacteria that when deposited on plants through its oral secretions, downregulate JA-induced defenses in tomato. Hosting these bacteria seems to be beneficial for the beetle because downregulation of JA-related defenses increased insect performance (Chung et al. 2013). Beetles do not possess salivary glands, therefore their oral secretions mainly arise from their gut as regurgitant. Lepidopteran larvae, on the other hand, release secretions from both their salivary glands (saliva) and from their gut as regurgitant to different degrees (Felton et al. 2014). Much work has been done to characterize molecules present in the lepidopteran oral secretions that regulate induction of plant defenses while neglecting the role that microbes may play in these interactions (Felton and Tumlinson 2008).

Lepidopterans constitute one of the largest phytophagous insect groups, comprising about 180,000 described species some of which are important agricultural pests (The Lepidoptera Taxome Project http://www.ucl.ac.uk/taxome). Surprisingly, studies of their gut microbiome are scarce, comprising only a few species, including *Pieris rapae* (Robinson et al. 2010), *Spodoptera littoralis* (Tang et al. 2012), *Helicoverpa armigera* (Priya et al. 2012) *Spodoptera exigua* (Ping et al. 2007), *Anticarsia gemmatalis* (Vistto et al. 2009), *Plutella xylostella* (Indiragandhi et al. 2008) and *L. dispar* (Broderick et al. 2004). Gut-associated bacteria of lepidopterans can promote plant growth (Indiragandhi et al. 2008), produce digestive proteases (Vistto et al. 2009), metabolize toxic plant compounds (Mason et al. 2016), and appear to help in the hydrolysis or synthesis of N-acyl amino acid conjugates, which are elicitors of JA-mediated plant defenses (Ping et al. 2007). These studies suggest a potential role of gut microbes in plant utilization and plant defense regulation in Lepidopteran, which has not been explored thus far.

In this study, we investigated the effects of bacteria isolated from the oral secretions of the polyphagous herbivore fall armyworm (FAW), *Spodoptera frugiperda* (Lepidoptera: Noctuidae) on herbivore-induced defenses in two host plants, tomato and maize. We used culture-dependent methods to identify microbes associated with field-collected FAW caterpillars and tested the direct effect of these bacteria isolates on plant JA-mediated defense responses. We also tested the indirect effect of some

of these isolates on activity and relative abundance of insect salivary proteins.

RESULTS

Identification of FAW associated gut bacteria and their effect on herbivore-induced defenses in tomato and maize plants

We tested the effect of FAW caterpillar gut bacteria on JA-mediated plant defenses in tomato and maize plants following the procedures reported in Chung et al. (2013) with some modifications. Field-collected caterpillars were either pretreated with antibiotics or untreated controls before allowing them to feed on plants. To ensure that all plants received the same amount of damage, the caterpillars were placed in clip cages and removed after they ate the enclosed leaf area. To test the effect of bacteria from caterpillars oral secretions in the regulation of JA-mediated plant defenses, plants were mechanically wounded and treated with 20 μl of regurgitant-diluted 1:100 in MQ water obtained from field-collected caterpillars that were either pretreated with antibiotics or untreated controls. Bacteria present in the regurgitant of FAW caterpillars modulated JA-related defense responses in tomato and maize, but in different ways. In tomato, feeding by field-collected caterpillars treated with antibiotics induced significantly higher activity of the defense-related protein polyphenol oxidase (PPO) than the untreated larvae ($t = 2.71$, $p = 0.018$, $n = 8$) (Fig. 1a). In maize, antibiotic-treated caterpillars induced similar transcript accumulation of the JA defense-related gene maize proteinase inhibitor (*mpi*) than the untreated caterpillar controls ($t = 0.83$, $p = 0.428$, n=6) (Fig. 1b). The same pattern of defense induction observed with caterpillar feeding was found when plants were treated with regurgitant from these caterpillars (Fig. 1c-d).

To identify bacteria present in regurgitant of field-gathered caterpillars, we collected secretions directly from the caterpillars oral cavity, diluted it 1:300 in sterile Milli-Q water, and plated 100μl of this mixture on sterile 2xYT agar plates. Individual bacterial colonies were further sub-cultured on 2xYT plates; from those, 15 bacteria samples (randomly picked) were used for identification using the Bruker Biotyper matrix-assisted laser desorption ionization-time of flight mass spectrometry, MALDI-TOF MS (Bruker Daltonics, Billerica, MA) system and 16S rRNA gene sequencing. We identified five different bacterial genera in the family Enterobacteriaceae (Table 1). An isolate of *Pantoea* sp. was further identified as *Pantoea ananatis* using specific primers (Figueiredo and Paccola-Meirelles 2012). These gene sequences were deposited in GenBank and accession numbers are listed in Table 1.

To identify which bacteria from the FAW oral secretions were inducing defense responses, we applied individual bacterial isolates to mechanically wounded plants. Each isolate was grown overnight on 2xYT liquid media and plants were treated with 20 μl (OD600 = 0.1 $\sim$ 9.0 X 10^6 Colony Forming Units CFU/ml) of it. This dose was within the range of the concentration of bacteria cultured from field-collected caterpillars (between 7.4 X 10^5 and 6 X 10^8; n = 14). The effect of each bacteria isolate on JA-inducible plant defense responses was compared with responses to application of liquid media alone. In tomato, two isolates, *Pantoea ananatis* and Enterobacteriaceae-1, suppressed the activity of the defense-related proteins PPO and trypsin protease inhibitor (trypsin PI), but induced greater peroxidase (POX) activity when compared with media-treated plants. *Raoultella* sp. and *Klebsiella* sp. had no effect on PPO, but suppressed POX activity and induced trypsin PI (Fig. 2 a-c). In maize plants, all bacteria isolates (except for *Enterobacter* sp.2 and Enterobacteriaceae-2) induced higher *mpi* gene expression than the liquid media (Fig. 2d). Additionally, the *P. ananatis* strain found in the FAW induced cell death in tomato leaves treated with bacteria, but this response was not observed on leaves treated with media only. No sign of cell death was observed in maize (Supplementary Fig. S1). Application of 2xYT growing media to wounded tomato leaves did not affect PPO activity compared to wounding alone ($F_{2,15} = 14.33$, $p = 0.000$, $n = 6$; Supplementary Fig. S2), indicating that the levels of defense responses observed in the 2xYT treatments were likely due to mechanical damage.

Plant defense responses triggered by FAW gut bacteria affected caterpillar growth

To test if defense responses elicited by bacteria from the FAW gut affect caterpillar growth, plants were treated with individual bacterial isolates and the leaves used to feed third-instar caterpillars 24 h later. Caterpillars fed on detached leaves from tomato plants treated with *P. ananatis* or Enterobacteriaceae-1 gained more weight than those fed on wounded tomato leaves treated with media only. These caterpillars gained the same amount of weight as larvae fed on leaves from unwounded control plants (Fig. 3a). In contrast, caterpillars gained less weight when fed maize leaves previously treated with *P. ananatis* and Enterobacteriaceae-1 (Fig. 3b). Caterpillars grown on leaves treated with *Enterobacter sp.*1 gained the same amount of weight as those fed on wounded leaves treated with media or untreated control plants.

FAW caterpillars secreted regurgitant onto plants during feeding

To verify that FAW caterpillars regurgitate while feeding on plants, we fed caterpillars with a florescent dye and quantified the amounts of regurgitant secreted on the feeding sites following a procedure previously described (Peiffer and Felton 2009). All caterpillars tested (n = 10) regurgitated on plants (Fig. 4). On average each caterpillar secreted 1.55 (± 0.460) nl and 3.29 (±0.290) nl of regurgitant per feeding bout on maize and tomato, respectively (Fig. 4).

Pantoea ananatis was secreted onto plants during FAW feeding

To confirm that *P. ananatis* was modulating JA-related plant defense responses observed following caterpillar feeding, this bacterium was cultured, diluted in MgCl2 solution, and reintroduced into antibiotic-treated caterpillars prior to placing them on plants. In tomato, caterpillars with reintroduced *P. ananatis* suppressed PPO activity in plants they were fed on compared to plants fed on by caterpillars without added bacteria (t = -3.08, p = 0.0132; n = 5-6) (Fig. 5a). In maize, expression levels of *mpi* were the same regardless of the presence of *P. ananatis* in FAW that fed on these plants (t = - 0.28, p = 0.779; n = 10) (Fig. 5b).
We verified that *P. ananatis* came into contact with damaged plant tissue during caterpillar feeding by doing PCR on the bacteria grown close to the feeding sites. *P. ananatis* was detected on 100% (n = 14) of the tomato plants and 60% (n = 10) of the maize plants fed on by caterpillars with re-introduced bacteria. *P. ananatis* was not found on plants fed on by caterpillar controls treated with antibiotics and MgCl2 solution (Fig. 5c-d).

FAW gut bacteria did not affect the activity of caterpillar salivary enzymes or the relative abundance of salivary proteins

In addition to regurgitant, FAW caterpillars also secrete saliva when feeding on plants (Chuang et al. 2014). Because this saliva contains components that induce JA-related plant defenses it is possible that the inoculation with bacteria may affect the caterpillar salivary composition. We tested the effect of *P. ananatis* and Enterobacteriaceae-1 on the activity of two salivary enzymes, GOX and PLC. The activity of these enzymes in salivary glands from caterpillars with reintroduced bacteria was not significantly different from the activity of caterpillar controls treated with buffer (Supplementary Fig. S3). We further tested if the presence of *P. ananatis* would affect the qualitative and quantitative abundance of salivary proteins of this insect. *P. ananatis* was reintroduced into last instar caterpillars and saliva collected for proteomic analysis using isobaric tags for relative and absolute quantification (iTRAQ); a control saliva sample was collected from caterpillars treated with antibiotics and supplemented with buffer. There were 4,936 spectra identified at the 95% confidence level out of 315,667 MS/MS spectra generated. From the spectra identified, 1840 were distinct peptides. The salivary protein abundances from caterpillars with reintroduced *P. ananatis* were not significantly different (qLFDR >0.05) from caterpillars treated with antibiotics and MgCl2 solution (Supplementary Table S1).

Bacteria from FAW were present in other insect species feeding on maize

We tested if the same bacterial taxa detected in the gut of FAW caterpillars were present in different insect species found in maize fields in 2014 and 2015. We identified bacteria from the regurgitant of the corn earworm, *Helicoverpa zea*, European corn borer, *Ostrinia nubilalis* and black cutworm, *Agrotis ipsilon*, larvae using 16S rRNA sequencing. We found *P. ananatis* in the regugitant of black cutworms, while Enterobacteriacea-1 and *Enterobacter sp.*1 were found in corn earworm larvae (Supplementary Fig. S4)

***P. ananatis* was found in FAW caterpillar frass**

During feeding, caterpillars deposit large amounts of frass that come into contact with wounded plant sites, which elicits plant defense responses (Ray et al. 2015). We tested if *P. ananatis* reintroduced into FAW could be detected in caterpillar frass. This bacterium was found on 60% of the samples tested (N = 10) (Supplementary Fig. S5).

Bacteria from FAW oral secretions did not affect caterpillar growth

We tested the effect of seven bacteria isolates from FAW regurgitant on the relative growth rate of young

caterpillars. Caterpillars grown on artificial diet containing bacteria gained the same weight as larvae fed on artificial diet without bacteria. There was only one bacteria isolate, *Raoultella* sp., that enhanced caterpillar growth (Supplementary Fig. S6).

DISCUSSION

Bacteria from the OS of FAW caterpillars modulated JA-mediated plant defenses in response to caterpillar feeding, which affected the performance of this insect on specific hosts. In tomato, wounded plants treated with either field-collected caterpillars or their regurgitant induced lower anti-herbivore defenses compared with caterpillars treated with antibiotics (Fig. 1a-c). Conversely, maize anti-herbivore defense responses were not different in plants fed on by caterpillars with or without their associated bacteria (Fig. 1b-d). These results suggest that in addition to fatty acid amino acid conjugates, proteolytic fragments and hydrolytic enzymes (Schmelz 2015), the oral secretions of lepidopteran caterpillars also contain microbes able to modulate JA-mediated defense responses in some plant species.

From the microbial community present in the oral secretions of wild FAW caterpillars, we identified five different bacteria genera belonging to the family Enterobacteriaceae, which are commonly associated with phytophagous insects (Sugio et al. 2015). Five out of seven of these bacterial isolates elicited defense responses in tomato and maize upon their application to wounded leaves (Fig. 2). Of these, *P. ananatis* and Enterobacteriaceae-1 (*Serratia/Rahnella*) suppressed herbivore defenses in tomato compared with controls treated with culture media. Similar results were obtained when plants were fed on by caterpillars reinoculated with cultured *P. ananatis* (Fig. 5a). These results confirm that plant JA-mediated defenses were in fact being regulated by the presence of at least one of these bacteria isolates. However, it is possible that other bacteria taxa, not identified by our methods, could have contributed to the observed plant defense responses. Our results also show that FAW caterpillars actively secreted regurgitant on their feeding sites (Fig. 4). Furthermore, *P. ananatis* was deposited onto the plants during caterpillar feeding (Fig. 1c-d). This suggests that bacteria from the FAW oral secretions can easily come in contact with plant wounds where they can directly modulate JA-mediated defense responses. Our results agree with a previous study in which bacteria associated with the phytophagous Colorado potato beetle, *Leptinotarsa decemlineata*, suppressed herbivore-induced defenses in tomato plants (Chung et al. 2013). But to our knowledge, this is the first report of this phenomenon in Lepidoptera.

The effect of FAW-associated bacteria on JA-related plant defense responses appears to be host plant-specific. In tomato plants *P. ananatis* and Enterobacteriaceae-1 suppressed herbivore defenses, but in maize these bacteria isolates along with *Enterobacter* sp.1, *Raoultella* sp. and *Klebsiella* sp., induced transcript accumulation of the protease inhibitor gene *mpi* (Fig. 2). This could be due to differences in signal transduction pathways or receptor mediated recognition between these two host plants. Interestingly, when maize plants were treated with either wild caterpillars or caterpillars with reintroduced bacteria, there was a trend for *mpi* induction, but this effect was not significantly different from controls (Fig. 1b and 4b). This could be explained by the low regurgitation rates of the FAW when feeding on maize (Fig. 4b), or adverse interactions of these bacteria with fragmented maize leaves inside the insects gut. It has been reported that diet type and their associated chemical defenses modify the composition of insect microbial communities in lepidopterans (Priya et al. 2012; Mason et al. 2015).

Members of the same bacteria genera identified in the oral secretions of FAW caterpillars have been reported in other Lepidoptera species, with the most common being *Pantoea* and *Enterobacter* spp. (Broderick et al. 2004; Robinson et al. 2010; Priya et al. 2012; Tang et al. 2012). The few studies of lepidopterans have shown a very low diversity in microbial composition of this group with some overlap of specific taxa (Sugio et al. 2015). The highly alkaline conditions of lepidopteran midguts may negatively correlate with levels of microbial diversity, and therefore may select for a few taxa able to tolerate these conditions (Engel and Moran 2013).

Some of the bacteria isolates associated with field-collected FAW were also found in the regurgitant of *H. zea* and *A. ipsilon* feeding on maize in the same field (Supplementary Fig. S4). This suggests that these bacteria may have been acquired from their common host plant. Studies in other lepidopterans show that a great proportion of larval gut bacteria are obtained from the host they feed on (Priya et al. 2012; Mason and Raffa 2014). However, the composition of gut bacterial communities is also influenced by the taxonomic group to which an insect species belongs (Colman et al. 2012), this may partially explain why none of the FAW associated bacteria were found in *O. nubilalis*, although the methods used in this study only partially surveyed the bacterial community of these insects. Furthermore, the effect of these bacteria on plant defense regulation may be different when associated with different caterpillar hosts. It has been shown that not all caterpillars secrete oral secretions during their feeding activity (Peiffer and Felton 2009), which may be explained by differences in their gut morphology; caterpillars that usually regurgitate seem to have larger crops and smaller midguts than the ones that do not (Grant 2006).

The mechanisms by which FAW-associated bacteria regulate JA-inducible plant defenses are unknown. Our results show that these bacteria 1) were present in the insect oral secretions, 2) regulated herbivore-induced defenses when applied to wounded plants, 3) were secreted

onto the plants by insect regurgitation, and 4) had no effect on either the activity or the protein abundance of FAW saliva. Therefore, our results suggest that either the bacteria themselves or bacteria-derived components are eliciting specific plant defensive pathways. In addition, FAW benefitted from its association with gut bacteria by downregulation of JA-mediated defenses in some plant hosts without an apparent effect on its growth and development (Supplementary Fig. S6).

Some strains of *P. ananatis*, one the defensive-suppressing bacteria in tomato plants, have been previously reported as pathogens of several plant species including maize and tomato (Coutinho and Venter 2009). The specific strain of *P. ananatis* found in FAW induced cell death in tomato and may be pathogenic (Supplementary Fig. S1). *P. ananatis* has an extraordinary capacity to adapt to different environmental conditions; for example, it can have an endophytic, epiphytic, pathogenic or symbiotic association with its host plants (Coutinho and Venter 2009). It can also be found as a symbiont in insect guts (Wells et al. 2002; Murrell et al. 2003) and as a pathogen to humans (De Baere et al. 2004). The ability of *P. ananatis* to live in an insect gut facilitates its transmission to host plants; for example, this bacterium is vectored by the tobacco thrips, *Franklin-iella fusca* (Wells et al. 2002). Insects are common vectors of plant pathogenic bacteria; for instance, *Pantoea stewarti*, the causal agent of Stewarts wilt disease in maize, is vectored by the corn flea beetle *Chaetocnema pulicularia*, possibly through their frass (Nadarasah and Stavrinides 2011). Although not tested in this study, *P. ananatis* could potentially be vectored by FAW caterpillars and transmitted through the insects oral secretions and possibly frass. Pathogenicity of *P. ananatis* and *P. stewarti* seems to be regulated by quorum-sensing (QS) signal molecules that activate the production of exopolysaccharides (EPS) and formation of biofilms leading to infection (Koutsoudis et al. 2006; Morohoshi et al. 2007). *P. ananatis* produces two QS molecules, N-acyl-L-homoserine lactone and N-(3-oxohexanoyl)-L-homoserine lactone (Morohoshi et al. 2007); from these, the former activates SA-mediated defense responses in tomato and *Arabidopsis thaliana* (Schuhegger et al. 2006; Schenk et al. 2014). FAW-associated bacteria are likely to be recognized by the plant as a MAMP (microbe associated molecular patterns), therefore, inducing pathogen defenses that downregulate herbivore defenses by crosstalk between signaling pathways.

We conclude that FAW gut-associated microbes regulate herbivore-induced defenses and enhance the insect performance on tomato plants. *P. ananatis* and Enterobacteriaceae-1 (*Serratia/Rahnella*) were two of the JA defense-suppressing bacteria identified in FAW oral secretions. These bacteria did not alter salivary protein abundance or the activity of insect salivary enzymes; therefore, they appear to directly regulate plant defenses upon their secretion through the insects regurgitant and perhaps frass. This study contributes to our understanding of the mechanisms of plant defense regulation by lepidopteran caterpillars.

METHODS

Insects

Fall armyworm caterpillars were collected in summer 2014 from susceptible non-Bt maize fields (*Zea mays* cv. Providence) at the Russell E. Larson Agricultural Research Center located at Rock Springs, PA. This colony was then maintained under laboratory conditions (Pennsylvania State University, University Park, PA) feeding exclusively on corn leaves for 14 generations.

Plants

Tomato plants (*Solanum lycopersicum* cv. Betterboy) were grown in Promix potting soil (Premier Horticulture), fertilized once with two grams of Osmocote plus (15-9-12, Scotts, Marysville, OH) and used for experiments when their 5th leaf was fully expanded. Maize plants (*Zea mays* cv. B73 inbred line) were grown in Hagerstown loam soil, fertilized once with three grams of Osmocote plus and used at their V8-V9 physiological stage. Plants were grown under glasshouse conditions (14:10 hours of light: dark) at the Pennsylvania State University, University Park, PA.

Identification of gut bacteria

Regurgitant for bacteria identification was obtained from field-collected caterpillars on the same day they were gathered from the field. The regurgitant was collected directly from the caterpillars oral cavity using a 200 μl pipette and diluted 1:300 in sterile MQ water, 100 μl of this mixture were plated on sterile 2xYT media; the media contained 0.016 g/ml of Bacto Tryptone (Becton Dickinson and Co. Sparks, MD), 0.01 g/ml of Bacto Yeast extract (Becton Dickinson and Co. Sparks, MD), 0.005 g/ml of sodium chloride (BDH), and 0.014 g/ml of Agar (Bioserv. Newark, DE). Cultures were incubated overnight at 27 oC. Individual bacterial colonies were sub-cultured on 2xYT agar plates; after 24 h individual colonies were randomly selected and grown overnight in 2xYT liquid media (without agar) at 27 C on a rotary shaker at 200 rpm. One volume of

each liquid bacteria culture was mixed with one volume of sterile 50% glycerol (EMD) and stored at -80 C for further use. Bacteria identification was carried out using two methods: the Bruker Biotyper MALDI-TOF MS (Bruker Daltonics, Billerica, MA) system and 16S rRNA gene sequencing.

For the Biotyper, bacteria isolates were prepared for analysis using a direct transfer method following a standard Bruker protocol (Schmitt et al. 2013). Briefly, individual colonies from overnight cultures (grown in 2xYT agar plates) were transferred onto a MALDI target plate using a wooden toothpick and allowed to dry; the cells were lysed by applying 1 μl of matrix solution [10 mg/ml of -cyano-4-hydroxycinnamic acid (HCCA)] in 50% aqueous acetonitrile containing 2.5% of trifluoroacetic acid. The matrix-analyte mixture was allowed to dry and the resulting samples were used for the MALDI Biotyper data acquisition. A bacterial test standard (BTS; Bruker Daltonics) was used for instrument calibration and as a positive control. Matrix blank spots were included in each analysis to ensure that the target plate was thoroughly cleaned and there was no carryover signal. MALDI mass spectra were acquired on a Bruker Ultraflextreme MALDI TOF/TOF mass spectrometer in the linear, positive-ion mode. Spectra were processed using a factory default processing method for the Biotyper application and searched against a Bruker Taxonomy library containing 5,627 cellular organisms entries using MALDI Biotyper version 3.1 software. Manufacturer-recommended cutoff scores were used for identification; scores $\geqslant$2.0 indicate identification to the species level, scores between 1.7 and 1.999 indicate identification to the genus level, and scores of <1.7 indicate no identification.

The 16S rRNA sequences were analyzed using BLAST against the nucleotide database of the National Center for Biotechnology Information (NCBI) (http://ncbi.nlm.nih.gov) and the Ribosomal Database Project Naive Bayesian rRNA (rdp) Classifier Version 2.1, at the 95% confidence threshold (http://rdp.cme.msu.edu) (Wang et al. 2007). The PCR reaction contained 0.4 M of each universal 16S rRNA primer (530 F 5'-GTG CCA GCM GCC GCG G-3' and 1392R 5'-ACG GGC GGT GTG TRC-3'), 12.5 μl of the GoTaq Green Master Mix (Promega), 2 μl of the overnight liquid-grown bacteria previously diluted 1:5 in sterile water, and 8.5 μl of MQ water for a total volume of 25 μl. The PCR conditions had an initial denaturation step of 5 min. at 95 C, followed by 30 cycles of 95 C for 1 min, 57 C for 30 sec, and 72 C for 1 min. and a final extension step of 7 min. at 72 C. The samples were cleaned up from remaining primers and nucleotides by incubating 5 μl of the PCR product with 2 μl of EXOSAP-IT (USB Corporation) at 37 C for 15 min, followed by a denaturation step at 80 C for 15 min to inactivate the EXOSAP-IT reagent. The PCR products were subjected to Sanger sequencing at the Pennsylvania State University Genomics Core Facility.

Effect of caterpillar gut bacteria on herbivore-induced plant defense responses

The effect of caterpillar gut bacteria on herbivore-induced defenses in tomato and maize plants was tested using different strategies. First, field-collected caterpillars were either pretreated with antibiotics (see below) or untreated controls before allowing them to feed on plants. These caterpillars were placed on plants using clip cages (polypropylene with metallic micromesh screen, 23 mm diameter and 18 mm height) to standardize the amount of damage. Second, mechanically wounded plants were treated with regurgitant obtained from field-collected caterpillars that were either pretreated with antibiotics or untreated controls. Third, mechanically wounded plants were treated with individual bacteria (OD600 = 0.1 $\sim$ 9.0 X 10^6 CFU/ml) isolated from the FAW regurgitant and grown overnight on 2xYT liquid media. The effect of each bacterial isolate on JA-related plant defense responses was compared with responses to application of liquid media. To identify if the 2xYT liquid media itself could induce plant defense responses, we tested its effect combining with wounding versus wounding alone. Finally, to test the effect of single bacteria isolates in combination with caterpillar feeding on JA-mediated plant defenses responses, individual bacteria cultures were reintroduced into caterpillars pre-treated with antibiotics (described below), which were then placed on plants. The leaf tissue (50-70 mg) around the feeding/damaged sites was harvested 24 and 48 hours later (for maize and tomato, respectively) in liquid nitrogen and stored at -80 C for further analysis. JA-related plant defense responses were measured by assessing the activity of defense-related proteins and quantifying the expression of a JA defense-related gene. In tomato plants, we measured the activity of PPO, trypsin PI, and POX) using biochemical assays (described below). In maize plants we quantified the relative expression of the *mpi* gene using quantitative real-time PCR (qPCR) (described below).

Antibiotic treatment and determination of bacteria dose

Caterpillars were treated with a cocktail of antibiotics containing 12.82 mg/ml of neomycin sulfate (MP Biomedicals. Santa Ana, CA), 64.1 mg/ml of aureomycin (Bioserv. Newark, DE, USA) and 3.85 mg/ml of streptomycin sulfate (Amresco. Solon, OH.

USA) diluted in MQ water. Twenty microliters of the antibiotic cocktail were deposited as small drops onto a piece of maize leaf (∼2.5 cm2), placed into a plate of agar and air dried for 3-4 hours. Each caterpillar was allowed to eat only one treated maize leaf piece. Untreated caterpillars were fed maize leaves treated with MQ water. The antibiotics dose used in these experiments was determined by feeding caterpillars with different concentrations of antibiotics; 24 hours later, one microliter of regurgitant was collected from each caterpillar (n = 10) and diluted in 300 μl of sterile water. From this mixture, 100 μl were plated on 2xYT agar plates and the number of bacterial colonies counted 24 hours later. The dose used above was chosen to produce no detectable bacterial colonies at the dilution tested.
To determine the concentration of bacteria to use in our experiments, regurgitant was collected from field-collected caterpillars and serial dilutions were plated on 2xYT agar plates to quantify the CFU/ml. Subsequent dilutions of cultured bacteria isolated from FAW were plated on 2xYT agar plates to determine a dilution that was comparable to the concentration of bacteria in the regurgitant of field-collected caterpillars.

Reintroduction of bacteria isolates into caterpillars

To study the effects of individual bacteria isolates on induction of JA-mediated plant defenses, single bacteria isolates were reintroduced into caterpillars that were later allowed to feed on plants. Last instar larvae were first treated with antibiotics (as indicated above) to clear their gut. The following day, caterpillars were fed twice with a small piece of artificial diet (0.1 g) containing 10 μl of bacteria (OD600 = 1.0 $\sim$ 9.0 X 10^6 CFU/ml) resuspended in 10 mM of MgCl2. The bacteria isolates were grown overnight in 2xYT liquid media, the bacteria suspension was centrifuged at 5,000 g for 10 minutes, and the pellet was re-suspended in MgCl2 solution (Chung et al. 2013). Control caterpillars were fed diet and MgCl2 only. After the diet was eaten (overnight), all caterpillars were transferred into a new cup and fed with an untreated 2.5 cm2 leaf pieces before placing them on the plants (2 - 3 h).

Effect of induced plant defenses on caterpillar growth

We evaluated the effect of plant defense responses elicited by bacteria isolates from the FAW gut on caterpillar growth. Plants were mechanically wounded and treated with 20 μl of either liquid media or individual bacteria cultures (OD600 = 0.1 $\sim$ 9.0 X 10^6 CFU/ml) grown over-night on 2xYT liquid media. These bacterial cultures were uniformly applied over the wounded area (28 mm2). After 24 and 48 h (for maize and tomato, respectively) the treated leaves were detached and used to feed 3rd instar FAW caterpillars for 6 days. The entire treated leaf of each plant was used to feed three caterpillars and their average weight gain used as an independent biological replicate for the statistical analysis. The caterpillar weight gain was calculated as the difference between initial and final weight.

Quantification of regurgitant on plant leaves

We quantified the amount of regurgitant secreted by the FAW caterpillars feeding on tomato and maize plants following the procedure described by (Peiffer and Felton 2009); caterpillars were grown from egg hatch on maize leaves and used when they reached their last instar. Ten micrograms of the fluorescent dye Alexa Fluor 488 (Invitrogen, Carlsbad, CA) were diluted in water and deposited onto the surface of leaves as small drops using a micropipette. These drops were allowed to dry for 3 h at room temperature in the dark; the leaf pieces were kept inside a petri dish containing 1% agar to avoid excessive dehydration. After the caterpillars ate the whole dye or water-treated leaf pieces, they were transferred to a new plastic cup and allowed to feed on fresh untreated leaves for about two min, and then the leaf pieces were used for detection of fluorescence. The amount of secreted regurgitant was measured using a standard curve.

Detection of specific bacteria on plant leaves

To verify that gut bacteria came into contact with plant wounds during caterpillar feeding, FAW caterpillars with reintroduced *Pantoea ananatis* were allowed to feed on plants for 30 60 min. The tissue around the feeding sites was harvested with clean scissors and forceps, placed into a sterile 2 ml tube containing 2xYT liquid media and incubated overnight at 27 C in a rotary shaker at 200 rpm. Caterpillars treated with antibiotics and fed with MgCl2 only were used as negative controls. The presence of *P. ananatis* in the bacteria culture was detected by PCR using specific primers developed by (Figueiredo and Paccola-Meirelles 2012). The PCR reaction contained 0.4 M of each primer (ANAF: 5'-CGT GAA ACT ACC CGT GTC TGT TGC -3' and EC5:

5'-TGC CAG GGC ATC CAC CGT GTA CGC T3'), 12.5 l of the GoTaq Green Master Mix (Promega), 2 μl of the overnight liquid-grown bacteria previously diluted 1:5 in sterile water, and 8.5 μl of MQ water for a total volume of 25 μl. The PCR conditions had an initial denaturation step of 5 min. at 95 C, followed by 35 cycles of 95 C for 1 min, 60 C for 30 sec, and 72 C for 1 min. and a final extension step of 5 min. at 72 C. The DNA fragments were separated on a 2% agarose gel run in 1X TAE at 75 V for 45 min, and then stained with SYBR green for visualization under UV light.

Protein activity assays

The activity of PPO and trypsin PI were measured as described previously (Chung and Felton 2011). The trypsin PI activity was calculated as PI (%) = (1-(slope of sample/slope of non-inhibitor))*100 and the resulting activity values normalized by the amount of protein (mg) or the amount of fresh leaf tissue (grams) contained in the sample. POX activity was assayed as described by Bi and Felton (1995) with minor modifications; 50 mg of leaf tissue were ground in liquid nitrogen and homogenized in 1.25 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 5% of cross-linked polyvinylpyrrolidone (PVP) (Alfa Aesar, Ward Hill, MA) and centrifuged at 11,000 g for 10 min at 4 C. Five microliters of the supernatant were mixed with 10 μl of 3% H2O2 and 190 μl of 3 mM guaiacol (MP Biomedicals). The change in absorbance was measured at 450 nm for 5 min. GOX activity in the caterpillar salivary glands was measured as described previously (Eichenseer et al. 1999) and adjusted for a microplate reader. PLC enzymatic assays followed a protocol described previously (Kurioka and Matsuda 1976; Le Chevalier et al. 2015) and adapted for a microplate reader.

Effect of bacteria on FAW salivary protein abundance

Saliva for proteomic analysis was collected from last-instar caterpillars with and without reintroduced *P. ananatis* (following the procedure described above). FAW caterpillars were chilled on ice for 45 min and immobilized within a metallic hairclip; as they warmed up, caterpillar saliva was collected using a micropipette tip (VWR cat No. 53509-015) under a dissecting microscope (Olympus SZ30). Two saliva samples, each containing 10 μg of saliva and 0.4 μg of protease inhibitor (Sigma P2714) diluted in 10 μl of MQ water, were used for proteomic analysis using iTRAQ. The protein samples were prepared following the Pennsylvania State University College of Medicine Mass Spectrometry and Proteomics Core Facility standard protocol [See supplementary methods full details] adapted from the manufacturer's instructions (Applied Biosystems, Carlsbad, CA).

Presence of *P. ananatis* in caterpillar frass

Along with saliva and oral secretions, caterpillars deposit frass on plants during feeding. We tested if bacteria present in the caterpillars regurgitant would also be present in the caterpillars frass. Caterpillars with re-introduced *P. ananatis* were fed maize leaves for 2 days; the caterpillars were transferred to clean cups twice and fresh leaves provided every 12 h. At the end of the second day, fresh frass pellets were collected and placed in 2 ml tubes with 1.5 ml of 2xYT liquid media and incubated overnight at 27oC in a rotary shaker at 200 rpm. The following day, 1 μl of the cultured bacteria was transferred into a new tube with 1.5 μl of sterile liquid media and incubated overnight under the same conditions described above. Two microliters of the bacteria (diluted 1:5) were used for the detection of *P. ananatis* using PCR.

Bacteria from FAW in other caterpillar species

We collected caterpillars feeding on insect-susceptible maize fields (cv. Providence) at the Russell E. Larson Agricultural Research Center, Rock Springs, PA. Bacteria from caterpillar regurgitant were cultured in the same way described above for FAW. The 16S region was amplified from individual bacteria cultures, using the same primers described above, and sequenced. The sequences of bacteria genera corresponding to those found in the FAW were aligned using Clustal 1.6 with a gap opening penalty of 15 and a gap extension penalty of 6.66; the resulting sequence alignment was used to construct phylogenetic trees using the UPGMA hierarchical clustering method. The test of phylogeny was done with the bootstrap method with 1,000 replications using the software MEGA 5.0 (Tamura et al. 2011).

Pathogenicity of *P. ananatis* to maize and tomato plants

Plant leaves were infiltrated with liquid cultures of *P. ananatis* (OD600 = 0.1 $\sim$ 9.0 X 10^6 CFU/ml) grown overnight on 2xYT media or liquid media alone. The plants were kept under greenhouse conditions for five days, time at which the leaves were detached from the plants, and photographed.

Effect of bacteria on caterpillar growth

We tested the effect of six bacterial isolates from FAW regurgitant on the relative growth rate of FAW caterpillars. Neonates were placed on artificial diet containing antibiotics [streptomycin (5 mg/100ml) and aureomycin (100 mg/100 ml)] for five days. Larvae were then transferred to new cups containing 0.8 g of artificial diet (without antibiotics) that had been inoculated with 50 μl of individual bacteria isolates diluted in 10 mM of MgCl2 (OD600 = 0.1 $\sim$ 9.0 X 10^6 CFU/ml). Controls received diet with MgCl2 solution alone. Fresh diet with bacteria was provided every two days in clean cups. The caterpillars were weighed two and five days later and their relative growth rate calculated as (W2 W1)/ [((W1+W2)/2)*d]; where W1 is the initial weight, W2 is the final weight and d is the number of days between measurements (Mohan et al. 2008).

RNA extraction, cDNA synthesis and real time PCR

Leaf tissue (50 - 70 mg) frozen in liquid nitrogen was homogenized in a GenoGrinder 2000 (OPS Diagnostics, USA) and total RNA was extracted using a modified Trizol protocol [See supplementary methods for full details]. Complementary DNA (cDNA) was synthesized from 1 μg of RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) using Oligo-dT following the manufacturers protocol. Quantitative real-time PCR (qRT-PCR) was conducted using the 7500 Fast Real-Time PCR System (applied Biosystems) with SYBR green (Roche Applied Science, USA) [See supplementary methods for full details]. The specific primers used for the target and reference genes (mpi and actin, respectively) were the same ones reported previously (Ray et al. 2015).

Experimental design and statistical analysis

The plant defense response (PPO, trypsin PI, POX activities and mpi gene expression) to different treatments (caterpillar feeding, application of caterpillar regurgitant and liquid bacteria isolates), the effect of bacteria reintroduction on the activity of GOX and PLC in the caterpillars salivary glands, and the effect of plant defense responses on caterpillar weight gain were analyzed with one-way ANOVA following the post hoc tests of Tukey and Fisher at α= 0.05. These statistical analyses were performed using Minitab 16 (Minitab Inc., State College, PA, USA) and all graphs were generated in R version 3.2.2 (Foundation for Statistical Computing, Vienna, Austria). The statistical analysis of quantitative iTRAQ were done using PSUTraq, which is an in-house modification of the MatLab program WHATraq developed by (Zhou et al. 2014). In PSUTraq, we added a Local False Discovery Rate calculation (qLFDR) based on (Storey and Tibshirani 2003) to correct for multiple testing. This qLFDR was calculated using the significant p-values (<0.05) obtained by Protein Pilot for the ratios of two samples with similar peak intensity values. Quantitative differences in the protein composition of saliva from caterpillars treated or untreated with *P. ananatis* were analyzed using log2 ratios of their peptide abundances.

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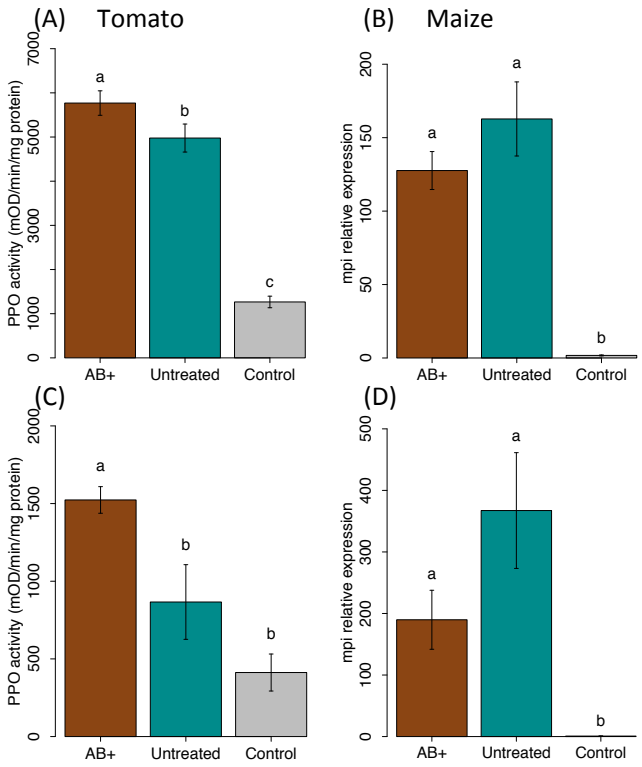

FIG. 1. Plant defense response to feeding and regurgitant treatment from field-collected fall armyworm caterpillars treated (AB+) or untreated with antibiotics. (A) Polyphenol oxidase (PPO) activity in tomato plants fed by caterpillars ($F_{2,25} = 106.10$, $p = 0.000$; $n = 8$ 11; Fisher test). (B) Maize Proteinase Inhibitor (mpi) gene expression in maize plants fed on by caterpillars ($F_{2,9} = 272.19$, $p = 0.000$; $n = 4$; Fisher test; log transformed data). (C) PPO activity in wounded tomato plants treated with caterpillar regurgitant ($F_{2,6} = 11.81$, $p = 0.0083$; $n = 3$; Fisher test). (D) mpi gene expression in wounded maize plants treated with caterpillar regurgitant ($F_{2,6} = 176.01$, $p = 0.000$; $n = 3$; Tukey test). Values are untransformed means SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at α=0.05. Controls are undamaged plants.

TABLE I. Bacteria isolates identified from fall armyworm oral secretions.

Gen Bank Accession No.	Name used in this paper	NCBI ID (% identity)	Rdp ID	Biotyper ID	Biotyper score
KX161909	*Pantoea ananatis*	*Pantoea ananatis (99%)*	Pantoea sp.	*Pantoea ananatis*	2.443
KX161910	*Enterobacter sp.1*	*E. cloacae* *E. ludwigii (99%)*	*Enterobacter sp.*	*E. asburiae* *E. ludwigii*	2.304 2.264
KX161911	Enterobacteriaceae-1	*Rahnella aquatilis* *Serratia quinivorans (99%)*	*Serratia sp.*	*Rahnella aquatilis*	2.099
KX161912	*Raoultella sp.*	*R. ornithinolytica (99%)*	*Raoultella sp.*	*R. ornithinolytica* *R. planticola*	2.363 2.362
KX161913	*Klebsiella sp.*	*K. oxytoca (97%)*	Enterobacteriaceae	*K. oxytoca*	2.322
KX161914	*Enterobacter sp.2*	*Enterobacter sp. (99%)*	*Enterobacter sp.*	*E. cloacae* *E. asburiae*	2.031 2.029
KX161915	*Enterobacteriaceae-2*	*E. ludwigii* *Pantoea sp.* *Pantoea dispersa (99%)*	*Enterobacteriaceae*	*Pantoea agglomerans**	1.816

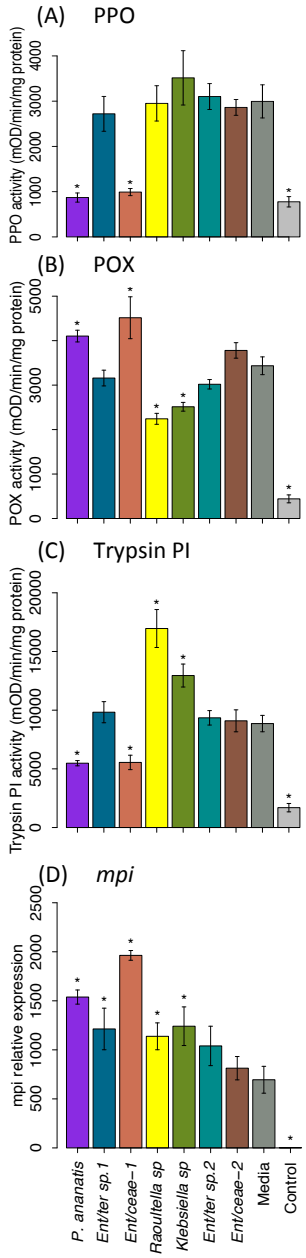

FIG. 2. Plant defense response to wounding plus the application of bacteria cultured from regurgitant of fall armyworm caterpillars. (A) Polyphenol oxidase (PPO) activity in tomato plants ($F_{8,64} = 16.74$, $p = 0.000$; n = 8 10; Fisher test). (B) Peroxidase (POX) activity in tomato ($F_{8,63} = 37.56$, $p = 0.000$; n = 8; Fisher test). (C) Trypsin proteinase inhibitor (Trypsin PI) activity in tomato ($F_{8,65} = 18.93$, $p = 0.000$; n = 5 10; Fisher test). (D) Maize Proteinase Inhibitor (mpi) gene expression in maize plants ($F_{8,50} = 183.94$, $p = 0.000$; n = 5 10; Fisher test; log transformed data). Values are untransformed means SEM; asterisks (*) indicate significant differences from media-treated plants obtained with ANOVA following post hoc tests at α=0.05. Ent/ter = *Enterobacter*, Ent/ceae = Enterobacteriaceae, controls are undamaged plants.

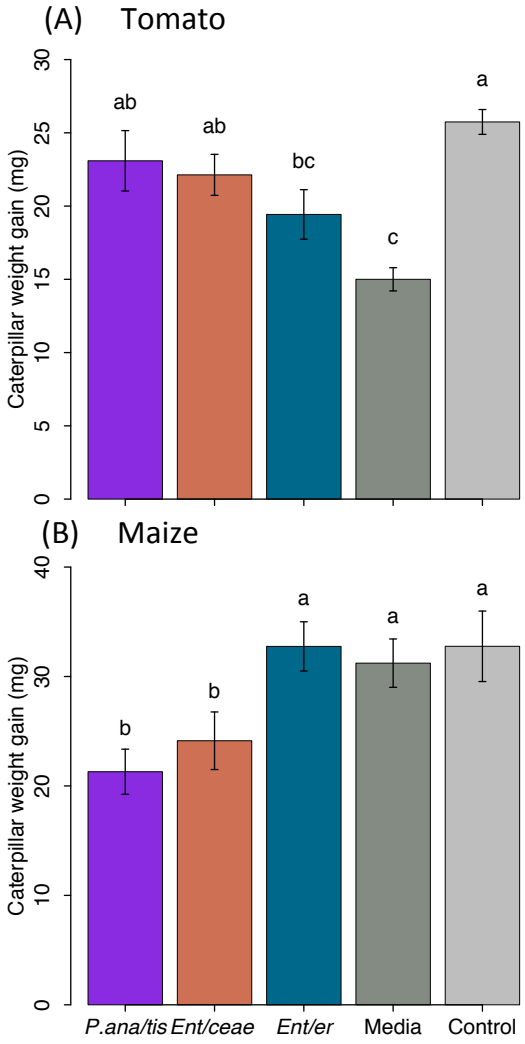

FIG. 3. Caterpillar weight gain after feeding on wounded plants treated with bacteria from fall armyworm regurgitant. (A) Weight gain (mg) of caterpillars fed on treated tomato plants ($F_{4,45} = 8.98$, $p = 0.000$; $n = 10$; Tukey test; log transformed data). (B) Weight gain (mg) of caterpillars fed on treated maize plants ($F_{4,25} = 6.24$, $p = 0.000$; $n = 4$ - 8; Fisher test; log transformed data). Values are untransformed means SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at α=0.05. Ent/aceae = Enterobacteriaceae-1, Ent/bacter = *Enterobacter* sp.1, controls are undamaged plants.

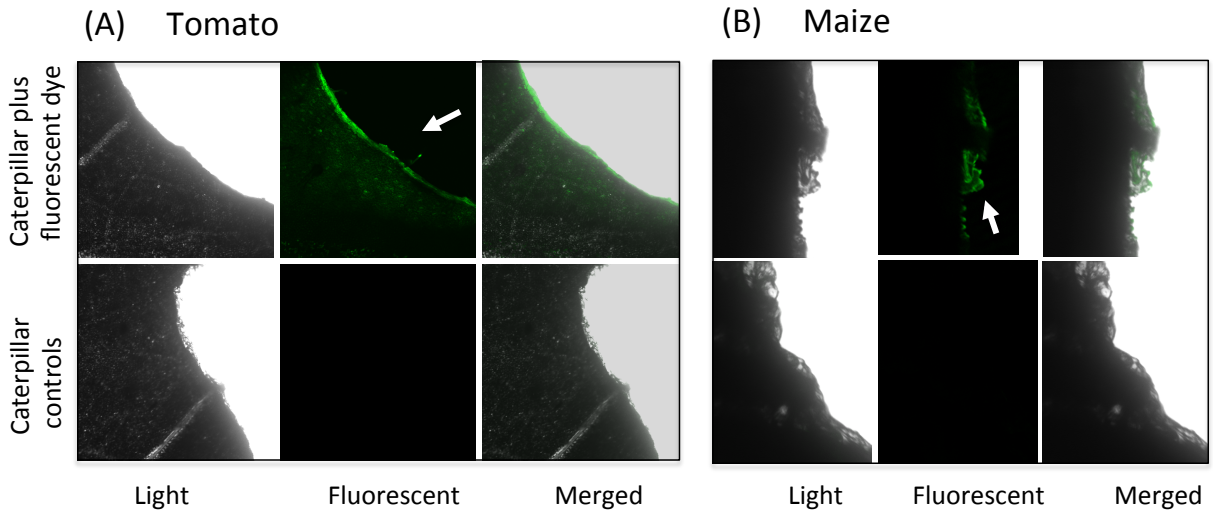

FIG. 4. Detection of regurgitant secreted by fall armyworm caterpillars on (A) tomato and (B) maize leaves using Fluorescent microscopy.

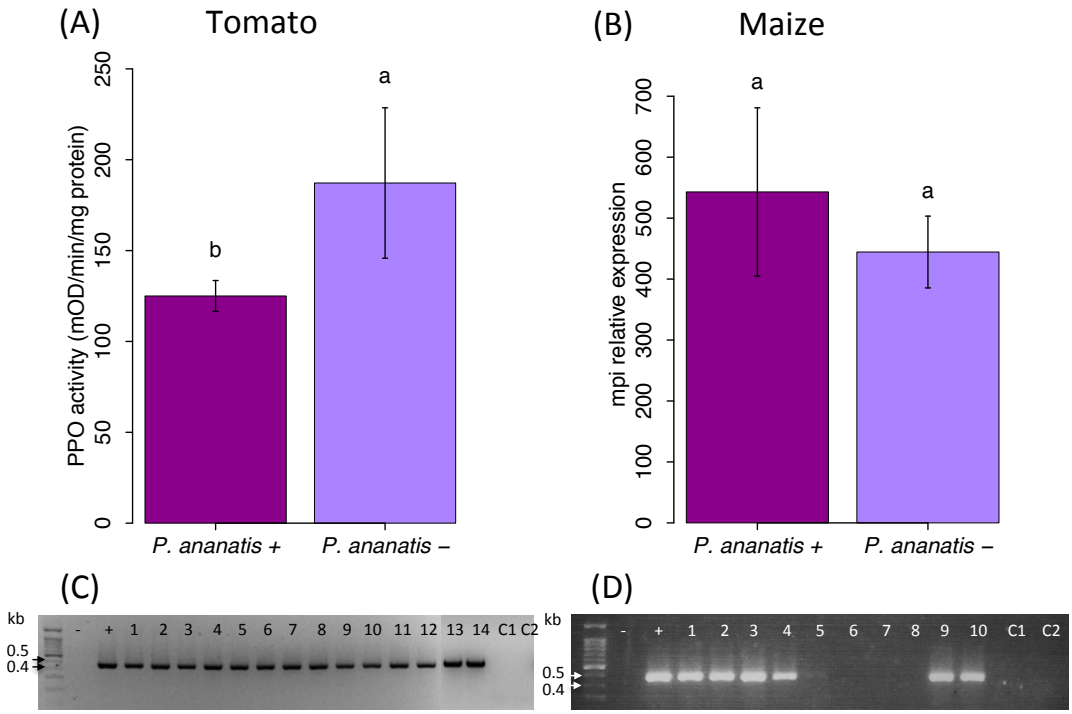

FIG. 5. Plant defense response to caterpillar feeding with (*P. ananatis* +) or without (*P. ananatis* -) reintroduced *P. ananatis* (A) Polyphenol oxidase (PPO) activity in tomato plants (t = -3.08, p = 0.0132; n = 5 6). (B) Maize Proteinase Inhibitor (mpi) gene expression in maize plants (F2,27 = 529, p ¡ 0.001; n = 10; Fisher test; log transformed data). (C) Presence of *P. ananatis* in tomato leaves fed on by caterpillars with reintroduced bacteria. (D) Detection of *P. ananatis* in maize leaves fed on by caterpillars with reintroduced bacterium. In the agarose gel images C and D, the negative and positive PCR controls, are depicted as () and (+) signs; lines 1-14 and 1-10 in C and D, respectively, are samples of regurgitant from caterpillars with reintroduced *P. ananatis*, while C1 and C2 are regurgitant samples from caterpillar controls. Bars are untransformed means SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at $\alpha = 0.05$. Controls are undamaged plants.