J Chem Ecol (2016) 42:193–201
DOI 10.1007/s10886-016-0677-z

Interactions Between Bacteria and Aspen Defense Chemicals at the Phyllosphere – Herbivore Interface

Charles J. Mason[1,2] · **Tiffany M. Lowe-Power**[3,4] · **Kennedy F. Rubert-Nason**[1] · **Richard L. Lindroth**[1] · **Kenneth F. Raffa**[1]

Received: 11 December 2015 / Revised: 17 February 2016 / Accepted: 20 February 2016 / Published online: 10 March 2016

Abstract Plant- and insect-associated microorganisms encounter a diversity of allelochemicals, and require mechanisms for contending with these often deleterious and broadly-acting compounds. Trembling aspen, *Populus tremuloides*, contains two principal groups of defenses, phenolic glycosides (salicinoids) and condensed tannins, which differentially affect the folivorous gypsy moth, *Lymantria dispar*, and its gut symbionts. The bacteria genus *Acinetobacter* is frequently associated with both aspen foliage and gypsy moth consuming that tissue, and one isolate, *Acinetobacter sp.* R7-1, previously has been shown to metabolize phenolic glycosides. In this study, we aimed to characterize further interactions between this *Acinetobacter* isolate and aspen secondary metabolites. We assessed bacterial carbon utilization and growth in response to different concentrations of phenolic glycosides and condensed tannins. We also tested if enzyme inhibitors reduce bacterial growth and catabolism of phenolic glycosides. *Acinetobacter sp.* R7-1 utilized condensed tannins but not phenolic glycosides or glucose as carbon sources. Growth in nutrient-rich medium was increased by condensed tannins, but reduced by phenolic glycosides. Addition of the P450 enzyme inhibitor piperonyl butoxide increased the effects of phenolic glycosides on *Acinetobacter sp.* R7-1. In contrast, the esterase inhibitor S,S,S,-tributyl-phosphorotrithioate did not affect phenolic glycoside inhibition of bacterial growth. Degradation of phenolic glycosides by *Acinetobacter sp.* R7-1 appears to alleviate the cytotoxicity of these compounds, rather than provide an energy source. Our results further suggest this bacterium utilizes additional, complementary mechanisms to degrade antimicrobial phytochemicals. Collectively, these results provide insight into mechanisms by which microorganisms contend with their environment within the context of plant-herbivore interactions.

Keywords *Acinetobacter* · *Lymantria dispar* · Condensed tannins · Detoxification · Gypsy moth · P450 · Phenolic glycosides

✉ Charles J. Mason
cjm360@psu.edu

[1] Department of Entomology, University of Wisconsin-Madison, Madison, WI 53706, USA

[2] Present address: Department of Entomology, The Pennsylvania State University, University Park, PA 16802, USA

[3] Microbiology Doctoral Training Program, University of Wisconsin-Madison, Madison, WI 53706, USA

[4] Department of Plant Pathology, University of Wisconsin-Madison, Madison, WI 53706, USA

Introduction

Herbivorous insects derive a wide range of benefits from microorganisms, such as enhanced nutrition (Gündüz and Douglas 2009; Scully et al. 2014), suppression (Chung et al. 2013), or degradation (Boone et al. 2013; Hammerbacher et al. 2013; Lauzon et al. 2003; Welte et al. 2015) of plant defenses, protection from natural enemies (Oliver et al. 2003), and pheromone synthesis (Xu et al. 2015). Relationships with nonpathogenic microorganisms can range from mutualistic to commensal, and from obligate to incidental (Douglas 2015; Oliver and Martinez 2014). Many of these studies have focused on highly stable systems where these microorganisms are transmitted vertically to offspring, as these provide the strongest opportunities for coevolutionary feedback and specialized mechanisms for maintaining symbiosis. However,

environmentally acquired microorganisms can likewise exert strong effects on herbivore success (Kikuchi et al. 2007; 2012), and such relationships are probably more numerous. We currently have less understanding of facultative and environmentally acquired symbioses. In particular, a key gap in our knowledge concerns interfaces between the various environments in which microorganisms reside. For example, bacteria that reside in plants or on plant surfaces encounter a radically different environment after their host plant is ingested by a herbivore. Bacteria that are consumed by herbivores become exposed to the macerated plant material, resident gut bacteria, and different pH and oxygen environments. Thus, foliar communities contribute to the structure of midgut communities, yet there are post-ingestive compositional changes influenced by the herbivore environment (Mason and Raffa 2014). Additionally, bacteria that remain on the foliage may become exposed to increased chemical concentrations due to defensive induction.

Microorganisms that reside in the environment have adapted various mechanisms to contend with plant allelochemicals. Detoxification mechanisms often are complex and multi-faceted, and can include multidrug efflux pumps (Del Sorbo et al. 2000; Nikaido 1996), catabolic degradation (Michielse et al. 2012; Singer et al. 2004), and inactivation by enzymatic addition of moieties (Matsuzaki and Wariishi 2004; Matthews and Etten 1983; Smith and Baker 2002). In many plant - microbe interactions, a pathogen's ability to neutralize phytochemical toxins is crucial to successful colonization and virulence (Lowe et al. 2015; Hassan and Hugouvieux-Cotte-Patte 2011; Michielse et al. 2012; Okmen et al. 2013; Tegtmeier and VanEtten 1982).

Several defense compounds mediate interactions between herbivores and *Populus* (Lindroth and St Clair 2013). The two major groups of defense chemicals in *Populus*, phenolic glycosides and condensed tannins, differentially affect insects of various taxa, host ranges, and feeding guilds (Barbehenn and Constabel 2011; Boeckler et al. 2011; Lindroth and St Clair 2013; Orians et al. 1997). Phenolic glycosides are distinguished by a glucose molecule bound to salicyl alcohol and β-D-glucopyranose moieties, with an ether linkage between the phenolic hydroxyl group and the anomeric C atom of the glucose. *Populus* produce phenolic glycosides with diverse chemical structures (Boeckler et al. 2011), with salicortin and tremulacin being the most prevalent in aspen (Lindroth et al. 1987). Concentrations of defense chemicals in *Populus* may increase in response to herbivory (Peters and Constabel 2002; Rubert-Nason et al. 2015).

Coadapted lepidopterans detoxify phenolic glycosides by cleaving them with esterases. Addition of chemicals such as S, S,S,-tributyl-phosphorotrithioate (DEF) and piperonyl butoxide to inhibit detoxification enzymes can increase Lepidoptera mortality to phenolic glycosides (Lindroth 1989; Lindroth and Hemming 1990). Additionally, gut conditions and ingested plant enzymes are considered to be mediators in the activation of and degradation of these compounds (Ruuhola et al. 2003). In comparison to phenolic glycosides, condensed tannins are larger and more structurally diverse compounds (Ayres et al. 1997; De Bruyne et al. 1999). Inactivation by lepidopteran herbivores is considered to be related to the alkalinity of the insect gut (Barbehenn et al. 2006; Barbehenn et al. 2009; Martin et al. 1985).

Populus defense chemicals have been shown to mediate several processes involving environmental pathogenic and commensal microorganisms. Condensed tannins can inhibit both bacteria and fungi (Scalbert 1991). Additionally, condensed tannins are correlated with antifungal activity in foliage (Bailey et al. 2005; Holeski et al. 2009) and influence soil microorganisms and litter decomposition (Bradley et al. 2000; Fierer et al. 2001; Madritch et al. 2007; Madritch and Lindroth 2011; Schweitzer et al. 2008). Our understanding of how phenolic glycosides can influence microorganisms is more limited than for condensed tannins, but bacteria in insect guts are influenced by phenolic glycosides in foliage and when incorporated into artificial diets (Mason et al. 2014, 2015).

Gypsy moth, *Lymantria dispar* L., is a polyphagous herbivore that exploits *Populus* among its most preferred hosts. Gypsy moth appears to acquire the majority of its symbiotic gut bacteria from environmental sources (Mason and Raffa 2014). Gut bacteria originating from aspen, *Populus tremuloides*, foliage can augment the ability of gypsy moth to contend with phenolic glycosides (Mason et al. 2014). Of the bacteria augmenting gypsy moth detoxification, a member identified as *Acinetobacter* exhibits the strongest community responses to phenolic glycosides. *Acinetobacter* are not always detected in colony gypsy moth sources, but are encountered when they consume aspen foliage containing these microorganisms (Mason et al. 2015). Interactions at the foliar - gut interface can be complex and nuanced. For example, aspen chemistry partially explains bacterial community composition of the larval midguts, but not of communities on the foliage (Mason et al. 2015). This pattern likely arises from the compartmentalization of defenses within intact foliage, resulting in only minimal contact between phyllosphere bacteria and compounds such as phenolic glycosides. Lysis of plant tissue initiates contact between bacteria and chemical defenses, which may continue post-feeding either in the insect or on the foliage. In this study, we investigated how aspen chemical defenses directly affect an *Acinetobacter* strain isolated from gypsy moth consuming aspen foliage. We tested the ability of *Acinetobacter* to use these compounds as a sole carbon source, its response to varying concentrations, and potential mechanisms of detoxification.

Methods and Materials

Chemical Extractions, Bacterial Strains, and Media Extracts of phenolic glycosides and condensed tannins were obtained from aspen foliage. Phenolic glycosides were extracted from field-collected leaves from an early season flush of one to 2-yr. old trees, while condensed tannins were from fully-grown, mature trees. Crude extract procedures were performed according to the methods of Lindroth et al. (1987) and Hagerman and Butler (1980), for phenolic glycosides and condensed tannins, respectively. Extracts of phenolic glycosides were predominantly salicortin (40 %) and tremulacin (60 %). Other compounds used in this study were obtained from commercially available sources.

Bacterial strains were isolated from the midguts of laboratory-reared gypsy moth larvae feeding on aspen as previously described (Mason et al. 2014). Briefly, third instars were anesthetized by refrigeration, surface sterilized, and dissected. Tissues were homogenized in phosphate buffered saline (pH 7.4), diluted, and plated onto growth medium. Bacteria were identified by sequences of the 16S SSU rRNA gene. The strain used in our study, *Acinetobacter sp.* R7-1, was previously shown to degrade phenolic glycosides (Mason et al. 2014). All growth experiments were conducted using the same protocols and equipment, but various time points of growth were used in the analysis to incorporate day-to-day variation in culture conditions.

Bacterial strains were routinely recovered from frozen glycerol stocks for all assays. For experiments requiring minimal carbon availability, cultures were grown in Boucher's minimal liquid medium (BMM; per liter: 3.4 g KH_2PO_4, 0.5 g $(NH_4)_2SO$. 100 μl 1.25 mg mL^{-1} stock solution $FeSO_4$. $7H_2O$, 517 μl of 1 M $MgSO_4$ solution, pH 5.5) with the addition of 10 mM MES (2-(*N*-morpholino)ethanesulfonic acid) (Boucher et al. 1985). For all other experiments, cultures were grown in casamino acid–peptone–glucose (CPG; per liter: 5 g glucose, 10 g peptone, 1 g casamino acid, pH 7.4) liquid medium. CPG was selected as a medium because phenolic glycosides were stable over the course of the experiment (Mason et al. 2014). We conducted our experiments at neutral and acidic pH to minimize phenolic glycoside degradation. In experiments assessing culture growth, optical density at 600 nm was used as it has a strong relation to cellular growth and number of colony forming units in the culture.

Influence of Single Carbon Sources on Bacterial Growth Bacterial cultures were grown overnight (~18 h) from glycerol stocks in liquid CPG medium. Cells were pelleted by centrifugation, rinsed in BMM lacking a carbon source, and re-suspended in BMM without carbon to an $OD_{600\ nm}$ of 0.2. Aliquots (100 μl) of the cell suspension were added to a clear 96-well round-bottomed microplate. Cell suspensions were supplemented with glucose, succinate, and extracts of phenolic glycosides (dissolved in DMSO) and condensed tannins (dissolved in sterile water) to a total final concentration of 1.0 mg mL^{-1} in 200 μl. The microplate was incubated at 28 °C with shaking in a Bio-Tek Synergy HTX (Winooski, VT, USA) plate reader. Growth was quantified at 15 h post inoculation, and growth of each strain provided with the carbon sources was compared with a negative control containing cells, but lacking carbon source.

Effects of Varying Concentrations of Phenolic Glycoside and Condensed Tannin on Culture Growth Cultures of *Acinetobacter sp.* R7-1 were grown overnight (~18 h) from glycerol stocks in liquid CPG medium. Cells were pelleted by centrifugation, rinsed, and re-suspended in CPG to an $OD_{600\ nm}$ of 0.2. Aliquots (100 μl) of the cell suspension were added to a clear 96-well round-bottomed microplate. Cell suspensions were amended with crude extracts of phenolic glycosides or condensed tannins to final concentrations ranging from 0 to 5.0 mg mL^{-1}. DMSO and water controls were added to the 0 mg mL^{-1} treatment. The plate was incubated with shaking at 28 °C, and growth was quantified by optical density.

Influence of Detoxification Enzyme Inhibitors on Bacterial Degradation of Phenolic Glycosides To test effects of enzyme inhibitors on cell growth by *Acinetobacter sp.* R7-1, cultures were amended with phenolic glycosides and one of two enzyme inhibitors: a cytochrome P450 inhibitor, piperonyl butoxide (90 % technical grade, Sigma-Aldrich, St. Louis, MO, USA), or an esterase inhibitor, S,S,S,-tributyl-phosphorotrithioate (DEF) (97 %, Chem Service, West Chester, PA, USA). Treatments included: unamended culture suspensions, cultures containing 5.0 mg mL^{-1} phenolic glycosides, cultures containing 0.5 % v v^{-1} enzyme, and a combination of both 5 mg mL^{-1} phenolic glycosides +0.5 % v v^{-1} inhibitor. Cultures were grown at 28 °C with shaking.

Phenolic Glycoside Culture Catabolism and Chemical Analysis Because piperonyl butoxide interacted with phenolic glycosides to reduce cell growth of *Acinetobacter sp.* R7-1 (see Results), we conducted a separate experiment to determine the effects of varying phenolic glycoside concentrations and piperonyl butoxide on the degradative ability of *Acinetobacter sp.* R7-1. Cells were grown in overnight cultures, pelleted, rinsed, and re-suspended in liquid CPG medium as previously described. Degradation experiments were conducted as described previously (Mason et al. 2014). Briefly, bacteria were grown for 24 h at 28 °C with shaking (200 rpm) in 200 μl of media in 5 ml glass culture tubes amended with crude extracts at a rate of 2.5 mg mL^{-1} and 5.0 mg mL^{-1}. Piperonyl butoxide was added to cultures to total 0.5 % v v^{-1}. Bacterial cells were pelleted and supernatants were frozen at −20 °C until analysis.

Phenolic glycoside contents of the bacterial culture supernatants were analyzed using a Waters integrated Acquity I-Class (Milford, MA, USA) ultra-high performance liquid chromatography system with negative electrospray ionization single quadrupole mass spectrometry detection (UHPLC/MS) (modified from Abreu et al. (2011)). Samples (2 μl) were injected onto a Waters Acquity CSH C-18 column (2.1 × 100 mm, 1.7 μm) and separated at 40 °C on a gradient of water and acetonitrile acidified with 0.1 % formic acid, with a flow rate of 0.5 mL min^{-1}. The mass spectrometer was operated in negative ionization mode, with selective ion recording of the salicinoid-formate adduct. The mass spectrometer was operated under the following conditions: cone potential, 30 V; capillary potential, 2500 V; extractor potential, 3 V; RF lens potential, 0.1 V; source temperature, 120 °C; desolvation temperature, 250 °C; desolvation gas flow, 500 L h^{-1}; cone gas flow, 10 L h^{-1}; infusion rate, 5 μL min^{-1}; dwell time, 0.025 s. Calibrations were based on internal standardization by salicylic acid-d_6 (Sigma-Aldrich), using four-point (15–1500 mg L^{-1}), quadratic models for salicin, salicortin, and tremulacin. The phenolic glycoside degradation products benzoic acid and saligenin also were assessed and quantified. Benzoic acid, salicin, and saligenin calibration standards were purchased from Sigma-Aldrich, and the tremulacin and salicortin calibration standards were extracted from aspen foliage by liquid-liquid extraction and purified by normal phase liquid chromatography.

Statistical Analyses All statistical analyses were conducted in the R (v. 3.0.1) statistical programing environment. For assessing effects of sole carbon sources, cell growth data could not be transformed to meet normality assumptions. Therefore, global comparisons were conducted with a Kruskal-Walis test. Growth of *Acinetobacter sp.* R7-1 in response to phenolic glycoside concentrations met normality assumptions and was analyzed using a linear model. Likewise, data on growth of *Acinetobacter sp.* R7-1 in response to phenolic glycosides plus piperonyl butoxide or DEF met normality assumptions and were analyzed with an ANOVA using both phenolic glycosides and piperonyl butoxide as fixed effects. Effects of treatment on phenolic glycoside concentrations were analyzed using two different ANOVAs. Phenolic glycoside concentrations were transformed to square roots to help achieve normality. The first ANOVA included fixed effects being treatment (control, bacteria, or bacteria and piperonyl butoxide) and phenolic glycoside concentration. The second analysis analyzed the two concentrations separately with treatment (control, bacteria, or bacteria and piperonyl butoxide) as fixed effects. This second analysis was used to conduct pairwise comparisons in Table 1. Pairwise comparisons were conducted using Tukey's HSD using the R package "agricolae."

Results

Acinetobacter sp. R7-1 exhibited no growth on the unamended minimal media over the course of the experiment (Fig. 1). *Acinetobacter sp.* R7-1 grew on media amended with succinate and condensed tannins (overall $\chi^2 = 12.32$; $P = 0.015$). It did not grow on media amended with either glucose or phenolic glycosides.

Phenolic glycosides reduced the growth of *Acinetobacter sp.* R7-1 in rich CPG growth media in a dose-dependent manner (Fig. 2) ($F_{1,10} = 27.83$; $P < 0.001$). At 5 mg mL^{-1}, growth of this isolate was reduced by 41 % relative to the controls. Conversely, condensed tannins improved the growth of *Acinetobacter sp.* R7-1 by 15 % at the same dose ($F_{1,10} = 10.32$; $P = 0.009$).

Addition of the cytochrome P450 enzyme inhibitor, piperonyl butoxide, in the growth medium exacerbated the growth reduction by phenolic glycosides of *Acinetobacter sp.* R7-1 (Fig. 3a). Statistically significant terms in the ANOVA included phenolic glycosides ($F_{1,20} = 122.63$; $P < 0.001$), piperonyl butoxide ($F_{1,20} = 47.23$; $P < 0.001$), and their interaction ($F_{1,20} = 6.50$; $P = 0.019$). Pairwise comparisons revealed that there was no difference between control cultures and those amended with piperonyl butoxide. However, phenolic glycosides alone, and combined with piperonyl butoxide, significantly reduced growth. Compared with the controls, phenolic glycosides reduced bacterial growth by 38 %, while phenolic glycosides with the addition

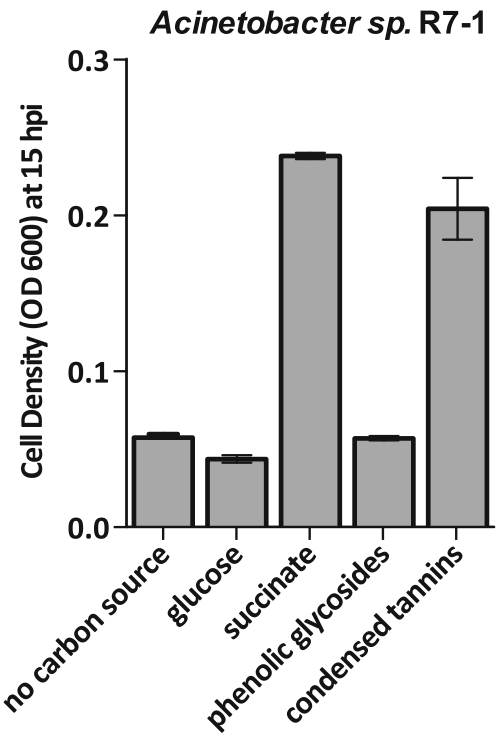

Fig. 1 Growth of *Acinetobacter sp.* R7-1 on Boucher's minimal medium (BMM) containing various sole carbon sources. Bars represent means ±1 se

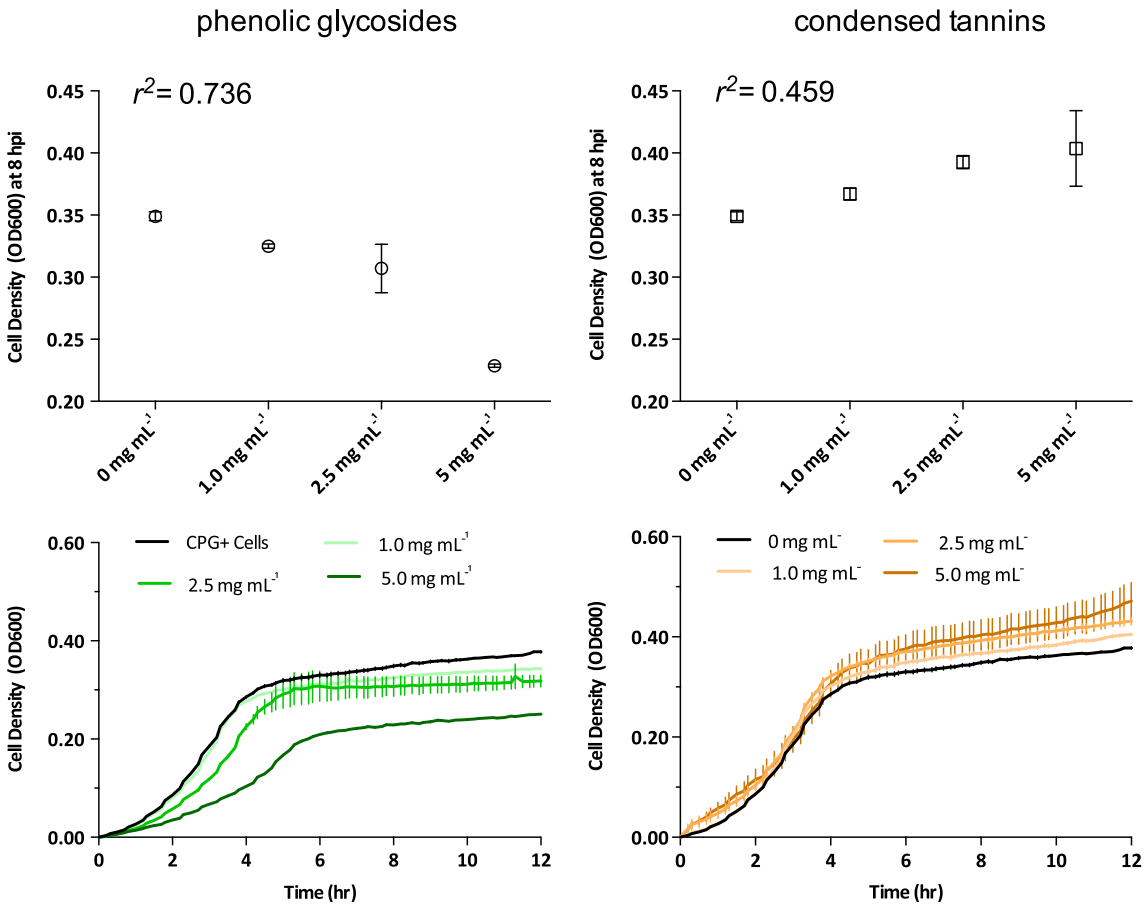

Fig. 2 Growth of *Acinetobacter sp.* R7-1 in response to increasing concentrations of phenolic glycoside and condensed tannins included in nutrient-rich medium. Bars represent means ±1 se

of piperonyl butoxide reduced bacterial growth by 71 %. In contrast, neither the esterase inhibitor DEF ($F_{1,8}$ = 0.931; P = 0.363), nor its interaction with phenolic glycosides ($F_{1,8}$ = 0.769; P = 0.406) influenced *Acinetobacter sp.* R7-1 growth (Fig. 3b).

Both starting concentration of phenolic glycosides and the presence of *Acinetobacter sp.* R7-1 influenced the amount of phenolic glycosides remaining in the growth media (Table 1). The presence of this bacteria reduced total phenolic glycosides ($F_{2,12}$ = 45.19 P < 0.001), salicortin ($F_{2,12}$ = 20.24; P < 0.001), and tremulacin ($F_{2,12}$ = 50.035; P < 0.001) compared with controls in both doses (2.5 and 5.0 mg mL^{-1}) in CPG. Additionally, *Acinetobacter sp.* R7-1 increased concentrations of the breakdown products salicin ($F_{2,12}$ = 24.15; P < 0.001),

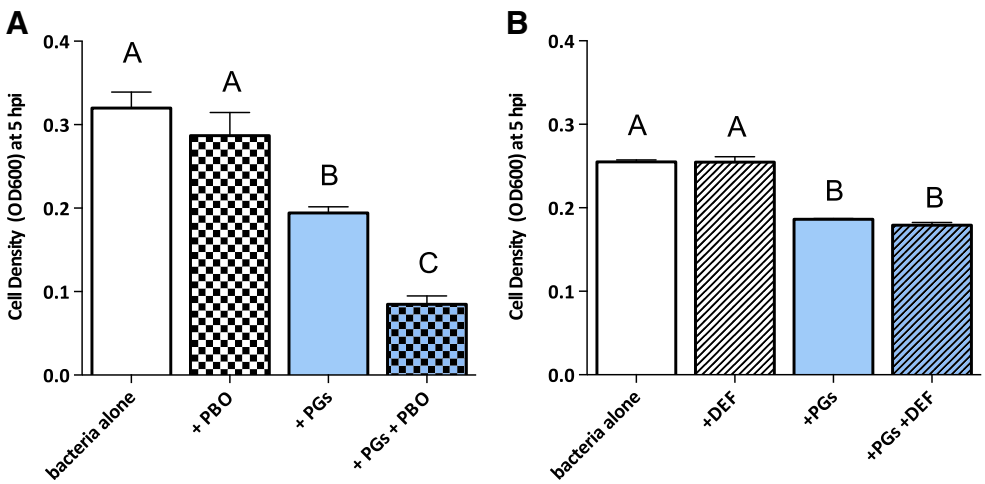

Fig. 3 Growth of *Acinetobacter sp.* R7-1 in response to piperonyl butoxide (PBO) (**a**), DEF (**b**) and phenolic glycosides (PGs) included in nutrient-rich medium. Bars represent means ±1 se. Letters represent statistically significant differences within an inhibitor treatment

Table 1 Phenolic glycoside degradation by *Acinetobacter sp.* R7-1 in nutrient-rich growth medium with and without piperonyl butoxide (PBO). Values are mean ± 1 se in mg. Numbers in italics are mean concentration in nmol. Letters correspond to significant differences (√y transformed data) between rows

	2.5 mg mL^{-1}			5.0 mg mL^{-1}		
	Control	*Acinetobacter*	*Acinetobacter* + PBO	Control	*Acinetobacter*	*Acinetobacter* + PBO
Total phenolic glycosides	1.524 ± 0.037 a	0.627 ± 0.086 b	0.840 ± 0.150 c	3.627 ± 0.143 a	2.025 ± 0.360 b	1.892 ± 0.433 b
	3.222	*1.635*	*2.069*	*7.627*	*4.556*	*4.286*
Salicortin	0.609 ± 0.040 a	0.289 ± 0.057 b	0.429 ± 0.085 c	1.410 ± 0.048 a	0.872 ± 0.193 b	0.843 ± 0.200 b
	1.436	*0.682*	*1.106*	*3.325*	*1.95*	*1.988*
Tremulacin	0.884 ± 0.003 a	0.208 ± 0.066 b	0.296 ± 0.081 b	2.153 ± 0.092 a	0.890 ± 0.281 b	0.855 ± 0.274 b
	1.674	*0.394*	*0.561*	*4.078*	*1.686*	*1.62*
Salicin	0.032 ± 0.003 a	0.130 ± 0.036 b	0.115 ± 0.030 b	0.064 ± 0.003 a	0.263 ± 0.118 b	0.194 ± 0.042 b
	0.112	*0.559*	*0.402*	*0.224*	*0.92*	*0.678*
Saligenin	0.014 ± 0.003 a	0.010 ± 0.000 a	0.012 ± 0.004 a	0.015 ± 0.001 a	0.010 ± 0.000 b	0.010 ± 0.000 b
	0.113	*0.081*	*0.097*	*0.121*	*0.081*	*0.081*
Benzoic acid	0.010 ± 0.000 a	0.045 ± 0.004 b	0.045 ± 0.005 b	0.019 ± 0.015 a	0.043 ± 0.005 b	0.043 ± 0.004 b
	0.069	*0.313*	*0.313*	*0.132*	*0.299*	*0.299*

benzoic acid ($F_{2,12}$ = 34.28; $P < 0.001$), and saligenin ($F_{2,12}$ = 7.76; $P = 0.002$). There was a significant effect of initial concentration on the amount of phenolic glycosides detected, but no interaction was present with bacterium. Reductions in the amounts of salicortin and tremulacin caused by *Acinetobacter* sp. R7-1 were greater when these compounds were administered at 5 mg mL^{-1} than 2.5 mg mL^{-1}. Results from ANOVAs of the separated concentrations had similar patterns. Pairwise comparisons indicated that salicortin and total phenolic glycoside concentrations with *Acinetobacter sp.* R7-1 were significantly different from those with *Acinetobacter sp.* R7-1 and piperonyl butoxide at the 2.5 mg mL^{-1} concentration. Compared to the control at the 2.5 mg mL^{-1} dose, *Acinetobacter sp.* R7-1 reduced total phenolic glycosides by 59 %, while *Acinetobacter sp.* R7-1 only reduced the concentration by 45 %. The effects of piperonyl butoxide were not present at the 5.0 mg mL^{-1} dose as *Acinetobacter sp.* R7-1 with and without the inhibitor only decreased the concentration by 48 and 45 %, respectively.

Discussion

Allelochemicals can have broad effects on herbivores and symbionts, thus influencing their interactions with one another. Gypsy moth obtains consortia predominantly from consuming foliage on and in which the bacteria reside (Mason and Raffa 2014). Bacteria residing in these tissues do not readily come into contact with plant allelochemicals, but the maceration of these tissues releases the compartmentalized metabolites and enzymes. The interaction of environmentally acquired bacteria, plant chemicals, and internal conditions of the midgut ultimately structure the community residing in the gypsy moth midgut (Mason et al. 2015). We found that bacteria responded to aspen compounds differently when they were incorporated into growth medium, which may affect proliferation and establishment in the insect larval gut.

Bacteria had varying interactions with primary and secondary compounds of aspen. *Acinetobacter sp.* R7-1 utilized condensed tannins, and succinate, as sole carbon sources. However, this strain did not utilize either phenolic glycosides or glucose as a carbon source. Various strains of *Acinetobacter* are known to differ widely in their ability to utilize glucose (Jumi 1978; Nemec et al. 2011). *Acinetobacter sp.* R7-1 responded to secondary compounds amended to nutrient – rich medium in a pattern consistent with their interactions with the compounds as a sole carbon source. Condensed tannins increased *Acinetobacter* growth in nutrient-rich medium, while phenolic glycosides reduced growth. These results parallel work in other systems, in which microbial symbionts were negatively affected by a diversity of plant chemicals, often in a concentration-dependent manner (Adams et al. 2011; Bailey et al. 2005; Hammerbacher et al. 2013; Kopper et al. 2005; Lauzon et al. 2003). Phenolic glycosides and condensed tannins commonly have disparate relationships with herbivores and other microorganisms, with various organisms exploiting or being unaffected by one, but being inhibited by the other (Bailey et al. 2005; Holeski et al. 2009; Orians et al. 1997; Osier and Lindroth 2001).

The contrasting effects of aspen secondary metabolites on *Acinetobacter sp.* R7-1 growth suggest differing ecological interactions. Because *Acinetobacter sp.* R7-1 was unable to utilize glucose or phenolic glycosides as a carbon source, we hypothesize that this organism degrades phenolic glycosides

to reduce toxicity. The interactions with phenolic glycosides may extend to gypsy moth, as gut microbial communities enriched with *Acinetobacter* can increase growth of gypsy moth (Mason et al. 2014). As a symbiont transitions between environments, its requisite degradation of phenolic glycosides and utilization of condensed tannins may in some instances be exploited by a herbivore for its own benefit. In other cases, degradation may provide little or no benefit to the insect, such as the ability of *Acinetobacter sp.* R7-1 to utilize condensed tannins, which appear to have only minor effects on gypsy moth. The positive effect of condensed tannins on *Acinetobacter sp.* R7-1 growth is not predicted by previous findings of reduced *Acinetobacter* sequence relative abundance in the gypsy moth midgut (Mason et al. 2015). However, our experiments do not fully encompass midgut conditions, such as high pH, interactions with other microorganisms, and digested plant material, which may influence *Acinetobacter* interactions with condensed tannins. It also is possible that the isolate we evaluated does not fully capture the metabolic diversity *Acinetobacter* present in the system (Welte et al. 2015).

The effects of enzyme inhibitors on bacterial cultures varied. The esterase inhibitor DEF did not increase the growth-inhibiting activity of phenolic glycosides. Possible explanations include that DEF did not fully reduce esterase activity due to substrate affinity, that the isolate had compensatory production, or that *Acinetobacter sp.* R7-1 does not degrade phenolic glycosides through this mechanism despite its occurrence in other organisms. In contrast, the P450 inhibitor piperonyl butoxide exacerbated the negative effects of phenolic glycosides on bacterial growth. The presence and activity of P450-like enzymes have likewise been shown in other strains of *Acinetobacter* (Asperger et al. 1981, 1984). Although piperonyl butoxide enhanced the growth-inhibiting effects of phenolic glycosides, we again found mixed results regarding how the inhibitor affects the ability of *Acinetobacter sp.* R7-to degrade the defense compounds. At low doses, the inhibitor significantly reduced the amount of phenolic glycosides in the medium, but this was absent at higher doses. The effect of a higher dose of phenolic glycosides may have elicited increased enzyme production to contend with the increased compounds. Interestingly, while there was a greater amount of degradation of phenolic glycosides in the higher doses, the proportion of phenolic glycosides reduced by *Acinetobacter sp.* R7-1 compared to the control was similar at both doses.

Our results characterize interactions of bacteria with plant defense chemistry at the plant - insect interface. These results also add to our overall understanding of how microorganisms may serve as a source of detoxification enzymes to insect herbivores (Adams et al. 2013; Scully et al. 2013). Several components of this interaction deserve further attention. First, the pH conditions of this experiment (5–7.4) were selected to prevent disassociation of the compounds rather than to simulate natural gypsy moth gut conditions. The gypsy moth midgut is highly alkaline (> 9.0), a condition under which phenolic glycosides are typically unstable (Ruuhola et al. 2003). Therefore, methods to quantify influences of bacteria under experimental conditions that remain controlled but also simulate gypsy moth midguts need to be developed. Additionally, investigations regarding the breadth of *Acinetobacter* association and metabolic diversity within and among gypsy moth populations are needed. Understanding the extent to which interactions of this nature occur in other plant-herbivore systems, and how they vary between generalist vs. specialist herbivores, would likewise improve our overall understanding of trophic relationships.

Acknowledgments This research was funded in part by UW-Madison College of Agricultural and Life Sciences, USDA Hatch #WIS01598 awarded to Kenneth Raffa, and USDA NIFA AFRI Grant No. 2011-67013-30147 awarded to Richard Lindroth and Kenneth Raffa. Tiffany Lowe is funded in part by NIH National Research Service Award T32 GM07215 and by USDA NIFA AFRI Grant No. 2015-67011-22799. Critical comments by three anonymous reviewers improved the quality of the manuscript.

References

Abreu IN, Ahnlund M, Moritz T, Albrectsen BR (2011) UHPLC-ESI/TOFMS determination of salicylate-like phenolic gycosides in *Populus tremula* leaves. J Chem Ecol 37:857–870

Adams AS, Boone CK, Bohlmann J, Raffa KF (2011) Responses of bark beetle-associated bacteria to host monoterpenes and their relationship to insect life histories. J Chem Ecol 37:808–817

Adams AS, Aylward FO, Adams SM, Erbilgin N, Aukema BH, Currie CR, Suen G, Raffa KF (2013) Mountain pine beetles colonizing historical and naive host trees are associated with a bacterial community highly enriched in genes contributing to terpene metabolism. Appl Environ Microbiol 79:3468–3475

Asperger ONA, Kleber H, Carl VEB, Jena Z (1981) Occurrence of cytochrome P-450 in Acinetobacter strains after growth on n-hexadecane. FEMS Microbiol Lett 11:309–312

Asperger O, Naumann A, Kleber H (1984) Inducibility of cytochrome P-450 in *Acinetobacter calcoaceticus* by n-alkanes. Appl Microbiol Biotechnol 19:398–403

Ayres MP, Clausen TP, Maclean SF, Redman AM, Reichardt PB, Reichardt PB (1997) Diversity of structure and antiherbivore activity in condensed tannins. Ecology 78:1696–1712

Bailey JK, Deckert R, Schweitzer JA, Rehill BJ, Lindroth RL, Gehring C, Whitham TG (2005) Host plant genetics affect hidden ecological players : links among *Populus*, condensed tannins, and fungal endophyte infection. Can J Bot 83:356–361

Barbehenn RV, Constabel C (2011) Tannins in plant-herbivore interactions. Phytochemistry 72:1551–1565

Barbehenn RV, Jones CP, Karonen M, Salminen J-P (2006) Tannin composition affects the oxidative activities of tree leaves. J Chem Ecol 32:2235–2251

Barbehenn RV, Jaros A, Lee G, Mozola C, Weir Q, Salminen J-P (2009) Hydrolyzable tannins as 'quantitative defenses': limited impact against *Lymantria dispar* caterpillars on hybrid poplar. J Insect Physiol 55:297–304

Boeckler GA, Gershenzon J, Unsicker SB (2011) Phenolic glycosides of the Salicaceae and their role as anti-herbivore defenses. Phytochemistry 72:1497–1509

Boone CK, Keefover-Ring K, Mapes AC, Adams AS, Bohlmann J, Raffa KF (2013) Bacteria associated with a tree-killing insect reduce concentrations of plant defense compounds. J Chem Ecol 37:1003–1006

Boucher C, Barberis P, Trigalet A, Demery D (1985) Transposon mutagenesis of *Pseudomonas solanacearum*: isolation of Tn5-induced avirulent mutants. J Gen Microbiol 131:2449–2457

Bradley RL, Titus BD, Preston CP (2000) Changes to mineral N cycling and microbial communities in black spruce humus after additions of $(NH_4)_2SO_4$ and condensed tannins extracted from *Kalmia angustifolia* and balsam fir. Soil Biol Biochem 32:1227–1240

Chung SH, Rosa C, Scully ED, Peiffer M, Tooker JF, Hoover K, Luthe DS, Felton GW (2013) Herbivore exploits orally secreted bacteria to suppress plant defenses. Proc Natl Acad Sci U S A 110:15728–15733

De Bruyne T, Pieters L, Deelstra H, Vlietinck A (1999) Condensed vegetable tannins: biodiversity in structure and biological activities. Biochem Syst Ecol 27:445–459

Del Sorbo G, Schoonbeek H, De Waard MA (2000) Fungal transporters involved in efflux of natural toxic compounds and fungicides. Fungal Genet Biol 30:1–15

Douglas AE (2015) Multiorganismal insects: diversity and function of resident microorganisms. Annu Rev Entomol 60:17–34

Fierer N, Schimel JP, Cates RG, Zou J (2001) Influence of balsam poplar tannin fractions on carbon and nitrogen dynamics in Alaskan taiga foodplain soils. Soil Biol Biochem 33:1827–1839

Gündüz EA, Douglas AE (2009) Symbiotic bacteria enable insect to use a nutritionally inadequate diet. Proc Biol Sci 276:987–991

Hagerman AE, Butler LG (1980) Condensed tannin purification and characterization of tannin-associated proteins. J Agric Food Chem 28:947–952

Hammerbacher A, Schmidt A, Wadke N, Wright LP, Schneider B, Bohlmann J, Brand WA, Fenning TM, Gershenzon J, Paetz C (2013) A common fungal associate of the spruce bark beetle metabolizes the stilbene defenses of Norway spruce. Plant Physiol 162:1324–1336

Hassan S, Hugouvieux-Cotte-Patte N (2011) Identification of two feruloyl esterses in *Dickeya dadantii* 3937 and induction of the major feruloyl esterases and of pectate lyases by ferulic acid. J Bacteriol 193:963–970

Holeski LM, Vogelzang A, Stanosz G, Lindroth RL (2009) Incidence of Venturia shoot blight in aspen (*Populus tremuloides* Michx.) varies with tree chemistry and genotype. Biochem Syst Ecol 37:139–145

Jumi E (1978) Genetics and physiology of *Acinetobacter*. Annu Rev Microbiol 32:349–371

Kikuchi Y, Hosokawa T, Fukatsu T (2007) Insect-microbe mutualism without vertical transmission: A stinkbug acquires a beneficial gut symbiont from the environment every generation. Appl Environ Microbiol 73:4308–4316

Kikuchi Y, Hayatsu M, Hosokawa T, Nagayama A, Tago K (2012) Symbiont-mediated insecticide resistance. Proc Natl Acad Sci U S A 109:8619–8622

Kopper BJ, Illman BL, Kersten PJ, Klepzig KD (2005) Effects of diterpene acids on components of a conifer bark beetle – fungal interaction: tolerance by *Ips pini* and sensitivity by its associate *Ophiostoma ips*. Environ Entomol 34:486–493

Lauzon CR, Potter SE, Prokopy RJ (2003) Degradation and detoxification of the dihydrochalcone phloridzin by *Enterobacter agglomerans*, a bacterium associated with the apple pest, *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae). Environ Entomol 32:953–962

Lindroth RL (1989) Biochemical detoxication: mechanism of differential tiger swallowtail tolerance to phenolic glycosides. Oecologia 81:219–224

Lindroth RL, Hemming JDC (1990) Responses of the gypsy moth (Lepidoptera: lymantriidae) to tremulacin, an aspen phenolic glycoside. Environ Entomol 19:842–847

Lindroth RL, St Clair SB (2013) Adaptations of quaking aspen (*Populus tremuloides* michx.) for defense against herbivores. For Ecol Manag 299:14–21

Lindroth RL, Hsia MTS, Scriber JM (1987) Characterization of phenolic glycosides from quaking aspen. Biochem Syst Ecol 15:677–680

Lowe TM, Ailloud F, Allen C (2015) Hydrocinnamic acid degradation, a broadly concerved trait, prrotects *Ralsonita solanacearum* from chemical defenses and contributes to root colonization and virulence. Mol Plant-Microbe Interact 28:286–297

Madritch MD, Lindroth RL (2011) Soil microbial communities adapt to genetic variation in leaf litter inputs. Oikos 120:1696–1704

Madritch MD, Jordan LM, Lindroth RL (2007) Interactive effects of condensed tannin and cellulose additions on soil respiration. Can J For Res 37:2063–2067

Martin MM, Rockholm DC, Martin JS (1985) Effects of surfactants, pH, and certain cations on precipitation of proteins by tannins. J. Chem. Ecol 11:485–494

Mason CJ, Raffa KF (2014) Acquisition and structuring of midgut bacterial communities in gypsy moth (Lepidoptera: Erebidae) larvae. Environ Entomol 43:595–604

Mason CJ, Couture JJ, Raffa KF (2014) Plant-associated bacteria degrade defense chemicals and reduce their adverse effects on an insect defoliator. Oecologia 175:901–910

Mason CJ, Rubert-Nason KF, Lindroth RL, Raffa KF (2015) Aspen defense chemicals influence midgut bacterial community composition of gypsy moth. J Chem Ecol 41:75–84

Matsuzaki F, Wariishi H (2004) Functional diversity of cytochrome P450s of the white-rot fungus *Phanerochaete chrysosporium*. Biochem Biophys Res Commun 324:387–393

Matthews E, Etten HDVAN (1983) Detoxification of the phytoalexin pisatin by a fungal cytochrome P-450. Arch Biochem Biophys 224:494–505

Michielse CB, Reijnen L, Olivain C, Alabouvette C (2012) Degradation of aromatic compounds through the β-ketoadipate pathway is required for pathogenicity of the tomato wilt pathogen *Fusarium oxysporum* f. sp. lycopersici. Mol Plant Pathol 13:1089–1100

Nemec A, Krizova L, Maixnerova M, Der Reijden TJK V, Deschaght P, Passet V, Vaneechoutte M, Brisse S, Dijkshoorn L (2011) Genotypic and phenotypic characterization of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex with the proposal of *Acinetobacter pittii* sp. nov. (formerly *Acinetobacter* genomic species 3) and *Acinetobacter nosocomialis* sp. nov. (formerly *Acinetobacter*. sp 13TU). Res Microbiol 162:393–404

Nikaido H (1996) Multidrug efflux pumps of gram-negative bacteria. J Bacteriol 178:5853–5859

Okmen B, Etalo DW, Joosten MHAJ, Bouwmeester HJ, De Vos RCH, Collemare J, De Wit PJGM (2013) Detoxification of α-tomatine by *Cladosporium fulvum* is required for full virulence on tomato. New Phytol 198:1203–1214

Oliver KM, Russell JA, Moran NA, Hunter MS (2003) Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proceedings of the National Academy of Sciences of the United States of America, 100:1803–1807

Oliver KM, Martinez AJ (2014) How resident microbes modulate ecologically-important traits of insects. Current Opinion in Insect Science, 4:1–7

Orians CM, Huang CH, Wild A, Dorfman KA, Zee P, Dao MTT, Fritz RS (1997) Willow hybridization differentially affects preference and performance of herbivorous beetles. Entomol Exp Appl 83:285–294

Osier TL, Lindroth RL (2001) Effects of genotype, nutrient availability, and defoliation on aspen phytochemistry and insect performance. J Chem Ecol 27:1289–1313

Peters DJ, Constabel CP (2002) Molecular analysis of herbivore-induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase from trembling aspen (*Populus tremuloides*). Plant J 32:701–712

Rubert-Nason KF, Couture JJ, Major IT, Constabel CP, Lindroth RL, Major IT (2015) Influence of genotype, environment, and gypsy moth herbivory on local and systemic chemical defenses in trembling aspen (*Populus tremuloides*). J Chem Ecol 41:651–661

Ruuhola T, Julkunen-Tiitto R, Vainiotalo P (2003) *In vitro* degradation of willow salicylates. J Chem Ecol 29:1083–1097

Scalbert A (1991) Antimicrobial properties of tannins. Phytochemistry 30:3875–3883

Schweitzer JA, Madritch MD, Bailey JK, Leroy CJ, Fischer G, Rehill BJ, Lindroth RL, Hagerman AE, Wooley SC, Stephen C, Whitham TG, Schweitzer A, Bailey K, Fischer DG, Hart SC (2008) From genes to ecosystems: the genetic basis of condensed tannins and their role in nutrient regulation in a *Populus* model system. Ecosystems 11: 1005–1020

Scully ED, Geib SM, Hoover K, Tien M, Tringe SG, Barry KW, Glavina Del Rio T, Chovatia M, Herr JR, Carlson JE (2013) Metagenomic profiling reveals lignocellulose degrading system in a microbial community associated with a wood-feeding beetle. PLoS One 8:1–22

Scully ED, Geib SM, Carlson JE, Tien M, Mckenna D, Hoover K (2014) Functional genomics and microbiome profiling of the Asian longhorned beetle (*Anoplophora glabripennis*) reveal insights into the digestive physiology and nutritional ecology of wood feeding beetles. BMC Genomics 15:1096

Singer AC, Thompson IP, Bailey MJ (2004) The tritrophic trinity: a source of pollutant-degrading enzymes and its implications for phytoremediation. Curr Opin Microbiol 7:239–244

Smith CA, Baker EN (2002) Aminoglycoside antibiotic resistance by enzymatic deactivation. Curr Drug Targ - Molec Disord 2:143–160

Tegtmeier KJ, Vanetten HD (1982) The role of pisatin tolerance and degradation in the virulence of *Nectria haematococca* on peas: A genetic analysis. Phytopathol 72:608–612

Welte CU, de Graaf RM, van den Bosch TJM, den Camp HJMO, van Dam NM, Jetten MSM (2015) Plasmids from the gut microbiome of cabbage root fly larvae encode SaxA that catalyses the conversion of the plant toxin 2-phenylethyl isothiocyanate. Env Microbiol DOI. doi:10.1111/1462-2920.12997

Xu L, Lou Q, Cheng C, Lu M, Sun J (2015) Gut-associated bacteria of *Dendroctonus valens* and their involvement in verbenone production. Microb Ecol 70:1012–1023