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Disrupting *Buchnera aphidicola*, the endosymbiotic bacteria of *Myzus persicae*, delays host plant acceptance

Cristina R. Machado-Assefh[1,2] · Guadalupe Lopez-Isasmendi[1,2] · W. Fred Tjallingii[3] · Georg Jander[4] · Adriana E. Alvarez[1]

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Abstract *Myzus persicae Sulzer*, like almost all aphids, associates with the endosymbiotic bacterium, *Buchnera aphidicola*. Although the accepted function of *B. aphidicola* is to complete the aphid diet with nutrients such as essential amino acids and vitamins, there is evidence that the bacteria may participate in the plant–insect interaction. Moreover, bacterial proteins with potential effector action on the metabolism of the host plant have been identified in the saliva of *M. persicae*. However, the possible involvement of *B. aphidicola* in relation to host plant acceptance by aphids needs further investigation. The aim of this study was to evaluate the effect that the disruption of the *B. aphidicola–M. persicae* symbiosis has on aphid feeding behaviour and on the expression of aphid salivary genes. The antibiotic rifampicin was administrated to adult aphids through artificial diets to disrupt the bacterial primary endosymbionts. Comparisons were made with control aphids, feeding from diet without rifampicin, as well as normal aphids fed on radish plants. Differences were found in the feeding behaviour of aposymbiotic aphids, which had delayed host acceptance and problems during stylet penetration into host plants. It was also found that *B. aphidicola* disruption down-regulated the expression of the Mp63 salivary protein gene. Together, these results indicate that *B. aphidicola* plays a role in plant–aphid interactions. The validity of the use of artificial diets in plant–aphid studies is also discussed.

Keywords EPG technique · Aphid primary endosymbiont · Green peach aphid · Artificial diets · Aphid salivary genes

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✉ Adriana E. Alvarez
alvareza@natura.unsa.edu.ar

1 Facultad de Ciencias Naturales, Universidad Nacional de Salta (UNSa), Av. Bolivia 5150, 4400 Salta, Argentina

2 Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), CCT-Salta, Av. Bolivia 5150, 4400 Salta, Argentina

3 EPG Systems, Wageningen, The Netherlands

4 Boyce Thompson Institute for Plant Research, Ithaca, NY 14853, USA

Introduction

Microbial mutualistic symbionts are increasingly being considered as important players in plant–insect interactions (Frago et al. 2012). The green peach aphid, *Myzus persicae Sulzer* (Hemiptera: Aphididae), is a piercing-sucking insect that feeds from phloem sap and establishes an obligate symbiosis with the bacterium *B. aphidicola* (Munson et al. 1991). *B. aphidicola* is located in the aphid haemocoel within specialized cells called bacteriocytes (Buchner 1965) and, as a result of long co-evolution with the aphid, has a reduced genome and is completely dependent on the aphids' intracellular habitat (Shigenobu et al. 2000, 2001; Whitehead and Douglas 1993). The presence of *B. aphidicola* is crucial for *M. persicae* since it provides essential amino acids and vitamins that aphids cannot obtain in sufficient quantities from phloem sap (Douglas 1993; Douglas 1996, 1998; Prosser and Douglas 1991). There is also evidence of plant–insect interaction processes in which this endosymbiont plays a role. A single clone of *M. persicae* fed on different plant species had differing amounts of *B. aphidicola* proteins in its haemocoel (Francis et al. 2006), and a clone of *Macrosiphum euphorbiae*

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Thomas showed differences in abundance in *B. aphidicola* proteins when it was fed on a resistant host versus a susceptible host (Francis et al. 2010). Recently, it has been demonstrated that a protein from *B. aphidicola,* GroEL, is present in aphid saliva (Vandermoten et al. 2014) and also that it is involved in the plant–aphid interaction (Chaudhary et al. 2014, Elzinga et al. 2014). However, the involvement of *B. aphidicola* in relation to host plant acceptance by aphids needs further investigation.

To select a host plant, aphids penetrate plant tissue by inserting their specialized mouthparts, the stylets, through the cell walls of the epidermis and the mesophyll (Tjallingii and Hogen Esch 1993). During the probing and feeding process, the aphids salivate repeatedly, producing gelling and watery saliva. The gelling saliva gels around the stylets forming a sheath between cells (Miles 1999). The gelling saliva may limit the damage of the cells in the epidermis and mesophyll and reduce aphid contact with the extracellular defences of the plant. The watery saliva contains proteins that participate in plant–aphid interactions (Miles 1999; Cherqui and Tjallingii 2000; Tjallingii 2006; Will and van Bel 2006; Will et al. 2007, 2012). The salivary proteins not only facilitate sap uptake from phloem but also can be involved in the recognition of the aphid feeding by the plant (Bonaventure 2012; Elzinga and Jander 2013). There are increasing studies on the diversity of aphid salivary proteins (Ramsey et al. 2007; Harmel et al. 2008; Carolan et al. 2009; Cooper et al. 2010, 2011; Rao et al. 2013). Moreover, there are proteins in the saliva of *M. persicae* and pea aphids, *Acyrthosiphon pisum, Harris* that affect the metabolism of the host plant and might be involved in the host plant acceptance by aphids (Will et al. 2007; Pitino et al. 2011; Pitino and Hogenhout 2013; Nicholson et al. 2012). Mutti et al. (2008) demonstrated that a salivary protein is crucial in the feeding of *Acyrthosiphon. pisum*, on fava bean (*Vicia faba*), and De Vos and Jander (2009) found that *M. persicae* produces a salivary proteinaceous elicitor that induces defence responses in *Arabidopsis thaliana*. The salivary proteins of *M. persicae* MP10, MP42, MpC002, and Mp55 were shown to affect the plant responses to aphids (Bos et al. 2010; Elzinga et al. 2014).

It also has been proposed that endosymbiotic microorganisms might be involved in plant–aphid interactions, since plant responses to aphids show similarities with plant responses to pathogens (Zarate et al. 2007; Alvarez et al. 2013; Thompson and Goggin 2006; Zhu-Salzman et al. 2004). For example, when *M. persicae* feeds on *Arabidopsis thaliana* and *Solanum tuberosum* plants, it induces expression of pathogenesis-related genes (PR genes) and genes related to the salicylic acid pathway (SA) (De Vos and Jander 2009; Alvarez et al. 2013). Both processes are associated with resistance to biotrophic pathogens (Walling 2000). Although the accepted function of *B. aphidicola* is to provide nutrients that aphids cannot get in sufficient amounts from their exclusive diet of phloem sap, there is also evidence that *B. aphidicola* participates more directly in the plant–insect interaction (Chaudhary et al. 2014; Elzinga et al. 2014). However, the previously reported evidence comes from *in planta* overexpression or exogenous application of a purified *B. aphidicola* protein, since the removal of *B. aphidicola* with antibiotics has many drawbacks in the aphids' physiology and may confound the effects when the interaction is evaluated in vivo. In this sense, detailed information about the interaction when the symbiosis is interrupted is needed.

By means of the electrical penetration graph (EPG) technique, it is possible to study in detail the plant penetration by the aphid's stylets. Thus, this method constitutes a robust tool to study the plant–aphid inter- and intracellular interaction at plant tissue level. The EPG technique was introduced by McLean and Kinsey (1964) and further developed by Tjallingii (1978b, 1985, 1988). The EPG waveforms have been correlated with aphid activities as well as with tissue locations of the stylet tips (Tjallingii 1978b, 1988; Kimmins and Tjallingii 1985; Tjallingii and Hogen Esch 1993). This technique previously has been used to study the feeding behaviour of aposymbiotic *Acyrthosiphom pisum* aphids whose development was impaired by the amino acids depletion caused by the disruption of *B. aphidicola* from the first nymphal stage (Wilkinson and Douglas 1995). The objective was to study the aphid feeding, focusing on the nutritional physiology of aposymbiotic aphids, which had smaller body size and had different feeding behaviour than control aphids with symbionts. However, this prior analysis needs to be extended to study the plant–aphid interaction in the absence of *B. aphidicola* in adult aphids that completed their normal development before the depletion of the symbionts.

The aim of the present study was to investigate the plant–aphid interaction when the primary endosymbiont of aphids, *B. aphidicola,* is disrupted by antibiotic treatment. The aphid feeding behaviour and expression of salivary genes were studied. The hypothesis is that *B. aphidicola* plays a role in plant–aphid interaction and so the disruption of the bacteria likely has an effect in the plant–aphid interaction in relation to host plant acceptance by the aphid. In order to compare the interaction with and without the bacteria, an antibiotic treatment was used to experimentally disrupt *B. aphidicola* from *M. persicae*. This approach was used before to study the aphid–symbiont nutritional interaction (Wilkinson and Douglas 1995; Prosser and Douglas 1991) and the interaction between *B. aphidicola* and other symbiotic bacteria (Koga et al. 2007, 2003). The final aim of this work is to provide new insights into the complex interaction occurring between aphids and plants.

Materials and methods

Insects

Myzus persicae was reared on radish (*Raphanus sativus* L.). Aphids used in the EPG experiments came from a colony maintained at the Faculty of Natural Science, National University of Salta, Argentina. This colony was initiated from a single virginoparous apterous individual collected in field in 2009 and maintained on radish (cv. Early Scarlet Globe, www.guasch.com.ar). For the salivary gene expression experiment, the *M. persicae* G006 green clone was used. The colony was initiated from a single virginoparous apterous individual collected in Geneva, New York, and maintained at the Boyce Thompson Institute, Ithaca, NY (USA), on radish plants (Spring Radish cv. Topsi, www.gourmetseed.com). In both cases, colonies were reared in a climate chamber at 22 ± 2 °C, 30–40 % r.h., and L16:D8 photoperiod to induce parthenogenesis. A new colony was started every week, and newly moulted adult apterae were used for the experiments.

To produce aposymbiotic aphids (aphids whose *B. aphidicola* have been disrupted), recently moulted adults of *M. persicae* were treated with 50 µg ml^{-1} of rifampicin (Prosser and Douglas 1991; Koga et al. 2007) for 4 days. The antibiotic was administrated through artificial diets containing 150 mM amino acids, 500 mM sucrose, vitamins, and minerals, following the protocol modified by AE Douglas (*pers. communication*) after Prosser and Douglas (1992). Aphids were placed on the diet cages that consisted of plastic cylinders 3 cm height × 2 cm depth. The diet sachet (diet solution between two layers of Parafilm) was applied across the top of the cage, which had a mesh on the bottom. The control aphids were fed on artificial diets without the antibiotic in the same conditions as aposymbiotic aphids.

To evaluate the efficiency of the antibiotic treatment, the expression of a *Buchnera*-specific gene, *GroEL*, was used as marker and measured by RT-qPCR. The primers used were: forward TCGCAAAATCAGGAAAACCT and reverse ACGACGATCTCCAAATCCTG. The methodology used for RNA isolation and purification, cDNA synthesis, and qPCR was as detailed below in the salivary gene expression experiment.

Feeding behaviour of aposymbiotic *M. persicae*

The DC-EPG technique (Tjallingii 1985, 1988) was used to monitor feeding behaviour of apterous aposymbiotic aphids compared to control aphids and normal aphids. Four plants were placed in a Faraday cage, and the feeding behaviour of two aphids on each plant was recorded simultaneously for 6 h. Aphids were placed on the abaxial side of a radish leaf, which was nearly fully expanded. Before exposure to the plant, the aphid was attached to the electrode whilst immobilized by a vacuum-suction device. The electrode consisted of a 2- to 3-cm-long gold wire (diameter 20 µm), conductively glued (water-based silver glue) to the dorsum. The other end of the gold wire was attached to a 3-cm-long copper wire (diameter 0.2 mm) and connected to the input of the first head stage amplifier with a 1 giga-ohm input resistance and 50× gain. The plant electrode, a 2-mm-thick, 10-cm-long copper rod, was inserted into the soil of the potted plant and connected to the plant voltage output of the EPG device (Giga-8, manufactured by EPG Systems (http://www.epgsystems.eu/). The recording was started immediately after wiring the aphids, at 20 ± 2 °C, under constant light in the laboratory, and about 1 h after collecting the aphids from the diet cages. Signals of eight aphids, two per plant on each set up, were acquired and recorded. Aphids from each of the three treatments were randomly distributed in the rounds of recording. Data acquisition was performed by PROBE 3.0 software (Laboratory of Entomology, Wageningen University, The Netherlands), and for waveform analysis, the software Stylet v01.23 (EPG Systems, Wageningen, The Netherlands) was used.

Comparisons were made between three aphid treatments, aposymbiotic aphids (aphids treated with antibiotic to disrupt *B. aphidicola*), control aphids (aphids fed on diet without the antibiotic), and normal aphids (aphids fed on radish plants). For each treatment, only the aphids that showed activities in every one of the 6 h of evaluation were considered as valid replicates. In total, 17 replicates for aposymbiotic aphids, 18 for control aphids, and 10 for normal aphids were obtained.

EPG waveforms, waveform patterns, and variables

Within the EPG signals, first a distinction was made between probes, i.e. periods of stylet penetration and periods of nonprobing. Then for analysis within probes, six waveform events (1–6) were distinguished, considering only uninterrupted periods as an event. Waveforms are generally grouped in three behavioural phases related to plant tissue location of the stylet tips, i.e. pathway, phloem, xylem phase, respectively, each comprising one or more waveforms. The waveform events distinguished here were: (1) waveform event C, including the three overlapping waveforms A, B, and C, in which waveform A reflects the first electrical stylet contact with the epidermis, B reflects intercellular sheath salivation, and C reflects stylet penetration movements. Also, the distinct potential drop (pd) waveform pd was considered as part of event C. The pds

reflect brief intracellular stylet punctures. Within the phloem phase, two separate E waveforms occur, (2) E1, sieve element salivation, and (3) waveform E2, phloem sap ingestion with concurrent salivation. When phloem sap ingestion E2 occurs, it is always preceded by phloem salivation E1, but the E1 waveform may occur as single waveform, without a subsequent E2. Also, E1 events may occur intermittently, alternating with E2 events, called E1 fragments. Then there are two waveform events that could be considered as belonging to pathway phase as well: (4) waveform E1e, putative extracellular (watery) salivation, and (5) waveform F, derailed stylet mechanics (stylet penetration difficulties). Finally, waveform G (event 6) is the only waveform in xylem phase, representing active sap ingestion from xylem elements (Tjallingii 1990a). Waveform event variables per treatment (n replicates, Table 2) were characterized into five broad categories following the nomenclature of the list of EPG variables of Tjallingii (downloaded from www.epgsystems.eu): (1) mean number of times waveform events occurred per insect; (2) mean of the mean duration of waveform events or per insect; (3) mean maximum duration of a waveform event per insect; (4) mean time to the first occurrence per insect of a waveform event from the start of the experiment or the probe; and (5) number or percentage of aphids that shows a particular waveform per treatment, with special interest in the percentage of aphids performing sustained phloem ingestion (sE2: uninterrupted period of E2 longer than 10 min). These variables were calculated for each insect treatment using the Excel® workbook for automatic parameter calculation of EPG data by Sarria et al. (2009). A total of 63 variables were obtained from EPG analysis, and 38 were selected as the most representative (Table 2).

Salivary gene expression in aposymbiotic *M. persicae*

Genes encoding six abundant *M. persicae* salivary proteins (Harmel et al. 2008) were identified from cDNA sequence data (Ramsey et al. 2007). Information about the evaluated genes is presented in Table 1. The Mp63 protein was identified based on a single fragment. Expression of these genes was compared between the three aphid treatments, aposymbiotic aphids, control aphids, and normal aphids. Total RNA was isolated from the whole body of aphids using TriReagent (Ambion) and SV Total RNA Isolation System (Promega). To remove genomic DNA, total RNA was treated with RQ1 RNase-Free DNase (Promega) according to the manufacturer's instructions. DNA-free total RNA was converted into cDNA using oligo dT20 primers, 10 mM dNTPs, and Clontech SMART MMLV Reverse Transcriptase according to the manufacturer's instructions. The qRT-PCR analysis was done in 384-well plates and covered with transparent adhesive films, both of optical quality (Applied Biosystems, Forest City, CA, USA) with an ABI 7900HT Fast System Real Time PCR (Applied Biosystems). The synthesis of double-stranded DNA was monitored by using SYBER Green as a reporter molecule. Each reaction contained 3.3 µl of cDNA (1/10), 0.9 µl of Mili-Q H_2O, 0.4 µl of each primer at a concentration of 3 mM, and 0.5 µl of the SYBER Green Mix (Applied Biosystems). The following program was used for all PCRs: 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The primers used for qPCR are presented in Table 2. To analyse the expression of each gene, the cycle at which the fluorescence reaches a threshold or "threshold cycle" (Ct) was calculated using the Applied Biosystems program (version 2.3 for Windows XP SDS). Ct values were calculated as the change in the relative expression (fold change) of the genes of interest normalized with the reference gene *RPL7* (Nikoh et al. 2010) using calibration curves constructed for each gene, using a pool of cDNA from all treatments.

Statistical analysis

All statistical analyses were performed using Infostat Profesional v2011p (http://www.infostat.com.ar) (Di-Rienzo et al. 2011). Student's *t* test was used to evaluate differences in *GroEL* expression levels, comparing the average of each aphid treatment versus the control aphids at a significance value of $p < 0.05$. EPG variables were analysed individually for each aphid, and then an average was calculated for each insect for each treatment, to obtain means and standard errors of the mean (SEM). In the case of multiple events, the mean was calculated from the average of each insect. Individuals that had not shown a certain waveform did not contribute to the calculated variable, and thus, n was smaller than the total number of replicates per treatment (Table 2). The Kruskal–Wallis nonparametric analysis of variance at one way of classification was performed since the EPG variables did not meet the normal distribution assumptions. When a significant effect was detected, pair-wise comparisons between means of treatments were performed with the Conover test. Fisher's exact test was used to evaluate the significance in the difference in proportions of individuals performing each type of activity. The expression of the salivary genes was analysed with ANOVA, and when a significant effect was detected, the differences between the means were evaluated using the LSD (less significant difference) test proposed by Fisher, comparing each treatment average versus normal aphids at a significance value of $p < 0.05$.

Table 1 Sequences of specific primers for *M. persicae* salivary genes used for qRT-PCR in aposymbiotic aphids, control aphids, and normal aphids

Protein name	GenBank ID	Predicted function	*A. pisum* gene identifier	Primers	Reference
Mp56	EC388700.1	Retinol dehydrogenase II	ACYPI007265	Fwd-TCTTTCCGTGGGTAAAGA Rev-GCCAACGTCGATGGTTCTAT	Harmel et al. (2008)
Mp57	EC388952.1	Unknown	Not annotated	Fwd-CCCAAACCTATGCAAAAGGA Rev-TTTGGATCCGTTTCACCATT	Harmel et al. (2008)
Mp60	EC389958.1	Unknown	ACYPI56506	Fwd-GTGTTACTCGTGCGTCGTGT Rev-TACGACCCGGTGGTTACATT	Harmel et al. (2008)
Mp61	EC389075.1	NADH dehydrogenase	ACYPI008219	Fwd-TTACGTAAAATGCCCGAGGA Rev-ATTCTTCGGCTTGTCCACAG	Harmel et al. (2008)
Mp62	ES221969.1	AMP-dependent CoA ligase	ACYPI003714	Fwd-TGTGATTATCGCCACAGCTC Rev-CGGGATAGTTTTGGGTCTTG	Harmel et al. (2008)
Mp63	DW010534.1	Hydroxyacyl dehydrogenase	ACYPI005506	Fwd-CAACAAAAGCAGTGCCCATA Rev-TCGTTGACCATTTTCGTCAG	Harmel et al. (2008)
RpL7[a]	–	Ribosomal protein L7	ACYPI010200	Fwd-TGCCGGAGTCTGTACTCAAA Rev-CACGCGTTCTTACGTTCCT	Nikoh et al. (2010)

The predicted functions are based on sequence similarities

[a] Reference gene

Results

Antibiotic treatment

The effectiveness of the rifampicin treatment was evaluated by comparing the transcript abundance of a specific *B. aphidicola* gene, *GroEL*. After 4 days of treatment with 50 μgml^{-1} rifampicin, the reduction in the number of transcripts of *GroEL* was 93 % ($t = -7.57$, $p = 0.016$), relative to control aphids without antibiotic treatment. There were no differences in aphid weight between aposymbiotic aphids and control aphids ($t = -1.19$; $p = 0.270$).

Feeding behaviour of aposymbiotic *M. persicae*

Feeding behaviour on radish plants differed between aposymbiotic, control and normal aphids. During the phloem phase, aposymbiotic aphids showed a lower number of sustained E2 events (Table 2, variable 27) than normal aphids and control aphids ($H = 5.28$, $p = 0.05$). The time from the beginning of the probe to the first E2 (Table 2, variable 17) and time from beginning of the probe to sustained E2 (Fig. 1a, variable 18) were longer for aposymbiotic aphids than for control and normal aphids ($H = 8.73$, $p = 0.02$ and $H = 8.48$, $p = 0.02$, respectively). The normal aphids and control aphids had a shorter duration of the pathway period before the first salivation in the phloem (Table 2, variable 16) than aposymbiotic aphids ($H = 11.49$, $p = 0.03$). Aposymbiotic aphids had the longest time from the beginning of the probe to the first E1 (Table 2, variable 14, $H = 14.4$, $p = 0.001$). Aposymbiotic aphids had stylet penetration difficulties (F) compared to control aphids and normal aphids. More aposymbiotic aphids showed F events (Table 2, variable 38), and a higher number of F events per insect (Fig. 1b; Table 2, variable 34) compared to the control aphids and normal aphids (Fisher's exact test, $p = 0.001$ and $H = 19.48$, $p = 0.001$, respectively). The number of G events (Table 2, variable 29) was higher in aposymbiotic aphids than in control aphids ($H = 6.44$, $p = 0.01$). Normal aphids did not have G events.

Differences were also found between aphids fed on diet (aposymbiotic aphids and control aphids) in comparison with normal aphids. The number and the total duration of nonprobing events (periods with stylets not inserted in the plant; Table 2, variables 2 and 3) were higher and longer for aposymbiotic aphids and control aphids ($H = 9.23$, $p = 0.01$, and $H = 8.94$, $p = 0.01$, respectively) than for normal aphids. In contrast, the total duration of probing (Table 2, variable 6) was the shortest for aposymbiotic aphids and control aphids, compared to normal aphids ($H = 9.23$, $p = 0.01$). The number of C events and their total duration (Fig. 2a; Table 2, variables 7 and 8) were higher and longer, respectively, for normal aphids than for aphids fed on diets (aposymbiotic aphids and control aphids) ($H = 10.18$, $p = 0.01$ and $H = 15.54$, $p \ll 0.01$, respectively). The total duration and mean duration of pd

Table 2 Electrical penetration graph (EPG) variables resulting from 6-h monitoring of aposymbiotic, control, and normal *M. persicae*

Related to	Number	EPG variables	Unit	Aposymbiotic ($n = 17$)	Control ($n = 18$)	Normal ($n = 10$)	p
Nonprobing	1	Time to 1st probe, from experiment beginning	s	128.9 ± 12.8	278.6 ± 130.1	117.9 ± 22.4	0.44
	2	Number of nonprobing events	#	13.4 ± 2.6^{b}	18.9 ± 3.33^{b}	5.2 ± 1.2^{a}	0.01*
	3	Total duration of nonprobing	min	38.6 ± 8.1^{b}	43.2 ± 7.9^{b}	12.6 ± 5.6^{a}	0.01*
	4	Mean duration of nonprobing event	min	5.7 ± 3.2	3.2 ± 1.1	2.3 ± 0.5	0.22
Pathway (C) and cell puncture (pd) events	5	Number of probes	#	13.4 ± 2.6^{b}	18.7 ± 3.3^{b}	5.2 ± 1.2^{a}	0.01*
	6	Total duration of probing	min	321.4 ± 8.6^{a}	316.8 ± 7.9^{a}	347.4 ± 5.6^{b}	0.01*
	7	Number of C events	#	15.8 ± 2.7^{b}	21.2 ± 3.5^{b}	6.0 ± 0.0^{a}	0.01*
	8	Total duration of C	min	123.5 ± 18.0^{a}	203.5 ± 19.0^{a}	71.5 ± 20.5^{b}	0.001**
	9	Mean duration of C event	min	8.9 ± 1.2	21.1 ± 8.4	14.2 ± 3.3	0.45
	10	Number of potential drops (pd)	#	61.2 ± 8.9	88.9 ± 14.4	45.2 ± 7.7	0.20
	11	Total duration of pd events	min	7.0 ± 1.1^{b}	9.82 ± 1.6^{b}	3.3 ± 0.5^{a}	0.002**
	12	Mean duration of pd event	s	6.7 ± 0.3^{b}	7.1 ± 0.8^{b}	4.5 ± 0.1^{a}	0.001**
	13	Time to 1st pd event	s	246.0 ± 44.7^{ab}	330.5 ± 68.1^{b}	47.2 ± 14.3^{a}	0.001**
Phloem phase events	14	Time to 1st E1 from beginning of probe with E1	min	110.9 ± 33.4^{b}	25.9 ± 7.5^{a}	21.5 ± 3.3^{a}	0.002**
	15	Time to 1st E2	min	249.2 ± 28.7	221.7 ± 28.3	40.2 ± 26.4	0.28
	16	Duration of the shortest C event before E1	min	87.2 ± 35.7^{b}	14.1 ± 2.3^{a}	21.5 ± 3.3^{a}	0.003**
	17	Time from beginning of the probe to 1st E2	min	115.0 ± 37.0^{b}	33.7 ± 7.3^{a}	22.3 ± 3.2^{a}	0.02*
	18	Time from beginning of the probe to 1st sE2	min	69.3 ± 13.8^{b}	30.8 ± 8.5^{a}	23.5 ± 3.3^{a}	0.02*
	19	Number of E1 (single E1 + E1 fractions)	#	1.2 ± 0.3	2.6 ± 0.6	2.4 ± 0.7	0.24
	20	Total duration of E1 (single E1 + E1 fractions)	min	1.3 ± 0.3	6.5 ± 2.4	28.3 ± 26.9	0.1302
	21	Mean duration of E1 (single E1 + E1 fractions)	min	0.7 ± 0.1	1.3 ± 0.3	6.0 ± 5.3	0.2
	22	Aphids that perform E1	# (%)	12 (71)	12 (67)	10 (100)	0.06§
	23	Number of E2	#	0.9 ± 0.2	1.5 ± 0.4	2.0 ± 0.5	0.12
	24	Total duration of E2	min	100.6 ± 27.5^{a}	122.3 ± 31.3^{a}	262.8 ± 33.3^{b}	0.002**
	25	Mean duration of E2	min	68.8 ± 19.7^{a}	78.3 ± 26.1^{a}	186.2 ± 39.8^{b}	0.04*
	26	Total duration of E12 (with both E1 and E2)	min	101.6 ± 27.6^{a}	124.8 ± 31.1^{a}	264.7 ± 33.4^{b}	0.002**
	27	Number of sustained E2	#	0.7 ± 0.2^{a}	1.1 ± 0.3^{b}	1.7 ± 0.3^{b}	0.05*
	28	Aphids with sustained E2 (>10 min)	# (%)	8 (48)	10 (56)	10 (100)	0.74§
Xylem phase events	29	Number of G	#	1.5 ± 0.5^{b}	0.2 ± 0.1^{a}	0.0 ± 0.0^{a}	0.01*
	30	Total duration of G	min	80.9 ± 23.8	56.7 ± 36.9	0.0 ± 0.0	0.41
	31	Mean duration of G	min	41.6 ± 14.4	56.7 ± 36.9	0.0 ± 0.0	0.94
	32	Time to 1st E1 since beginning of G	min	112.2 ± 57.4	47.0 ± 0.0	–	0.99
	33	Aphids that perform G	# (%)	9 (50)	4 (24)	0 (0)	0.09§

Table 2 continued

Related to	Number	EPG variables	Unit	Aposymbiotic ($n = 17$)	Control ($n = 18$)	Normal ($n = 10$)	p
Derailed stylet mechanics	34	Number of F	#	1.8 ± 0.3^b	0.2 ± 0.1^a	0.2 ± 0.2^a	0.001**
	35	Total duration of F	min	106.4 ± 28.9	80.9 ± 62.8	1.9 ± 0.0	0.52
	36	Mean duration of F	min	73.9 ± 25.8	80.9 ± 62.8	1.00 ± 0.0	0.64
	37	Time to 1st E1 since beginning of F	min	144.2 ± 35.6	47.0 ± 0.1	–	0.47
	38	Aphids that perform F	# (%)	15 (83)	3 (17)	1 (10)	0.001**§

p values according to Kruskal-Wallis non-parametric ANOVA (* $p \le 0.05$ and ** $p \le 0.01$) followed by multiple comparison with Conover test, values in a row with different letters indicate significant differences at $p \le 0.05$

§ Fisher's exact test for aposymbiotic aphids and control aphids at $p \le 0.05$

events (Fig. 2b; Table 2, variables 11 and 12) were longer in aposymbiotic aphids and control aphids than in normal aphids ($H = 7.71$, $p = 0.012$, and $H = 19.5$, $p = 0.0001$, respectively). The time from the first probe to the first potential drop (pd, Table 2, variable 13) was longer for aphids fed on artificial diets than for normal aphids ($H = 9.18$, $p = 0.001$). The total duration of E2 (Fig. 2a; Table 2, variable 24) was shorter for aposymbiotic aphids and control aphids than for normal aphids ($H = 12.23$, $p = 0.002$). The total duration of E12 (E1 followed by E2; Table 2, variable 26) was shorter for aposymbiotic aphids than for control aphids and normal aphids.

Salivary gene expression in aposymbiotic *M. persicae*

The effect of the disruption of *B. aphidicola* in *M. persicae* saliva was studied by analysing expression of salivary genes by qRT-PCR. The expression of six genes (Table 1) was assessed in the three treatments, aposymbiotic aphids (fed on artificial diet + rifampicin), control aphids (fed on diet without antibiotic) and normal aphids (reared on radish) (Fig. 3). The expression of *Mp63* gene (predicted hydroxy acyl dehydrogenase) was significantly lower in aposymbiotic aphids, compared to control aphids and normal aphids ($F = 8.65$, $p = 0.01$). For gene *Mp61* (NADH dehydrogenase) the expression of control aphids was lower than that for normal aphids, and this reduction was even greater in the case of aposymbiotic aphids ($F = 6.39$, $p = 0.02$). For the *Mp56* gene (retinol dehydrogenase), no differences were found between treatments ($F = 3.43$, $p = 0.08$). For the *Mp60* gene (unknown function), the expression showed an increase in aphids fed on artificial diets (aposymbiotic aphids and control aphids) versus normal aphids ($F = 5.23$, $p = 0.03$). *Mp62* expression showed significant differences only for control aphids relative to normal aphids ($F = 11.27$, $p = 0.004$). For the *Mp57* gene, no differences were found between the treatments ($F = 1.07$, $p = 0.384$).

Discussion

The feeding behaviour of aposymbiotic *M. persicae* showed differences in comparison with control aphids and normal aphids when feeding on radish plants. The aposymbiotic aphids had longer pathway phase before the first salivation in the phloem than control aphids and normal aphids (Table 2, variable 16). The time from the beginning of the probe until the first phloem salivation was the longest for aposymbiotic aphids (Fig. 1a; Table 2, variable 14). The aposymbiotic aphids also needed more time from the beginning of the probe to start the ingestion

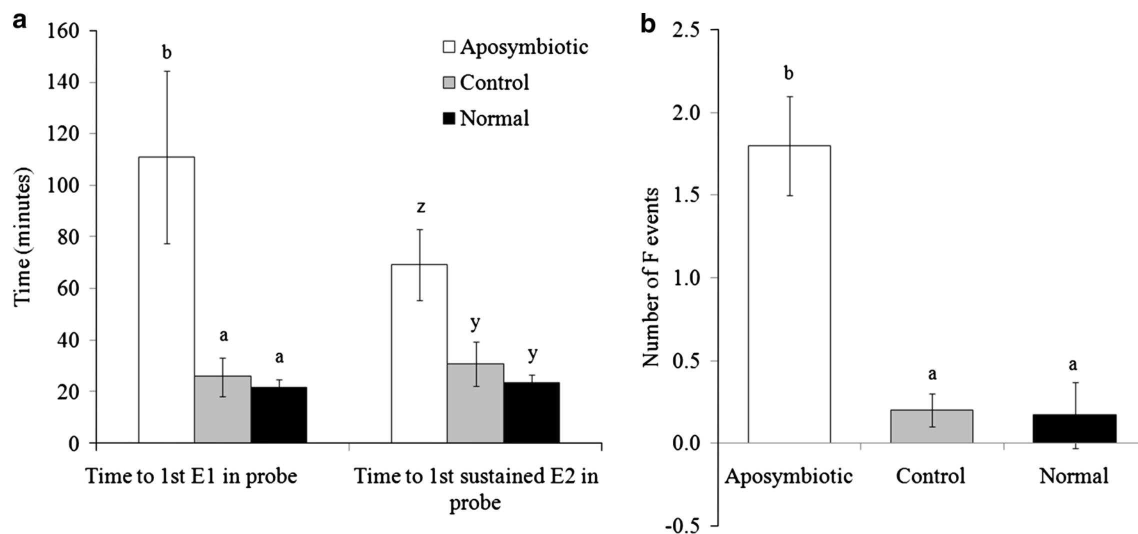

Fig. 1 Electrical penetration graph variables differing between aposymbiotic aphids versus control aphids and normal aphids, on radish plants. The histograms represent mean ± SEM. Within each variable, *different letters above bars* indicate significant differences (Conover test, $p \leq 0.05$). **a** Time from the beginning of the probe to first salivation (E1) and time from the beginning of the probe to first sustained ingestion (sE2). **b** Number of derailed stylet mechanics (F)

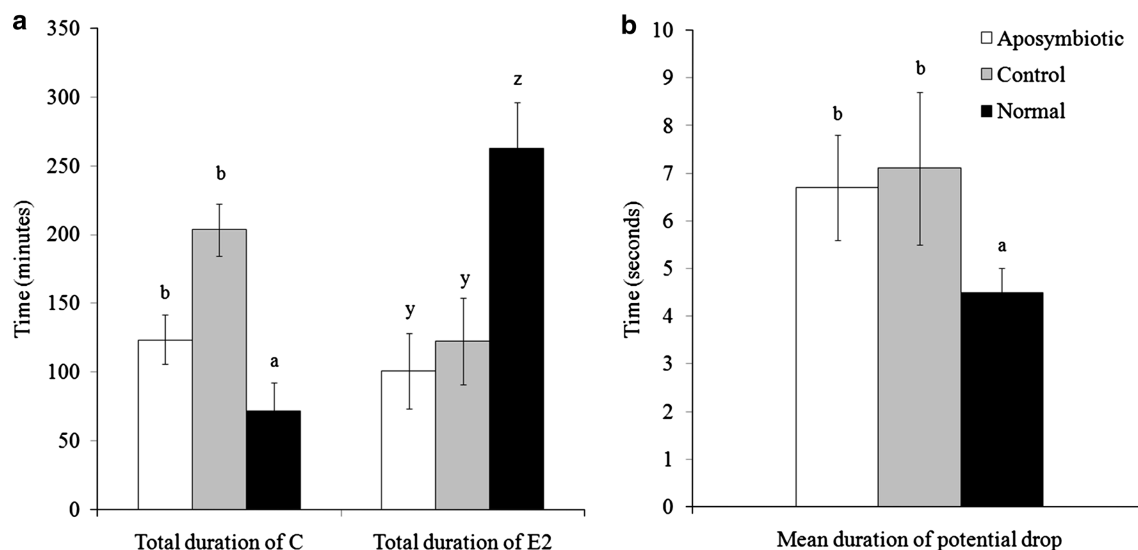

Fig. 2 Electrical penetration graph variables differing between diet-fed aphids (aposymbiotic aphids and control aphids) versus normal aphids, on radish plants. The histograms represent mean ± SEM. Within each variable, *different letters above bars* indicate significant differences (Conover test, $p \leq 0.05$). **a** Total duration of stylet pathway phase (C) and total duration of phloem sap ingestion (E2). **b** Mean duration of potential drops

of phloem sap (Table 2, variables 17). Furthermore, they needed more time to achieve sustained ingestion of phloem sap (sE2, longer than 10 min; Fig. 1a) than control aphids and normal aphids (Table 2, variable 18). The aposymbiotic aphids also showed a smaller number of sustained ingestion events (sE2) than control aphids and normal aphids (Table 2, variable 27). Phloem activities are the most important activities regarding plant acceptance since they determine the suitability of the plant as a host (Tjallingii 1990b). The salivation in the phloem always occurs first during sieve tube punctures, whether or not it is followed by phloem feeding, and once sustained feeding has been established, salivation likely allows continuous flux of phloem sap (Tjallingii 2006; Walling 2008). In addition, more aposymbiotic aphids showed waveform F and performed this activity more often as well (Fig. 1b; Table 2,

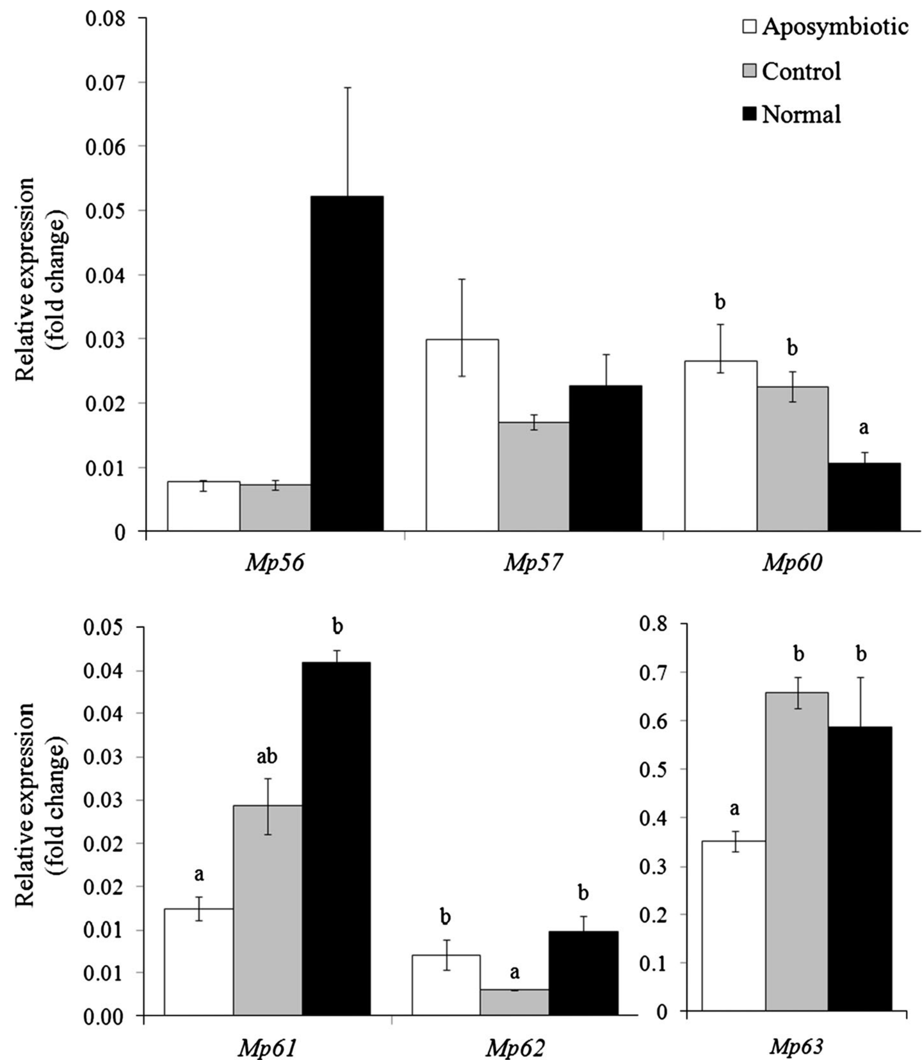

Fig. 3 Expression of *Myzus persicae* salivary genes in aposymbiotic aphids (fed on diets + rifampicin for 4 days to disrupt *B. aphidicola*), control aphids (fed on artificial diets without antibiotics), and normal aphids (fed on radish). The values are mean ± SEM of the expression of six salivary genes relative to the reference gene *RpL7*. *Different letters* indicate differences according to Fisher's LSD, $p \leq 0.05$

variables 34 and 38), which indicates problems with derailed stylet mechanics. These mechanical problems occur when an individual stylet lose their bundle formation with the rest of the stylets. The intercellular structure within plant cell walls seems to cause such derailments, but normally at a low frequency (Tjallingii 1978a). Here, the derailed stylet mechanics may be an indication that aposymbiotic aphids have penetration difficulties, probably due to differences in salivary components, but further studies are needed to explain this phenomenon. Together, these results suggest that plant acceptance was delayed in aposymbiotic aphids as compared to control aphids and normal aphids.

The second aim of this work was to assess the effect of disrupting *B. aphidicola* in *M. persicae* on the expression of the aphid salivary genes. Aphid saliva plays a fundamental role in the interaction with the host plants (Cherqui and Tjallingii 2000; Miles 1999; reviewed by Bonaventure 2012, and Elzinga and Jander 2013) and probably constitutes a link that would allow the interaction of *B. aphidicola* with the aphid's host plant. The abundance of transcripts of the salivary gene *Mp63* was significantly lower for aposymbiotic aphids than for control aphids and normal aphids (Fig. 3). This protein is a predicted hydroxyacyl dehydrogenase and so far has not been shown to have a role in the plant–insect interaction, since it did not affect *M. persicae* fecundity on *Nicotiana tabacum* (Elzinga et al. 2014). However, further investigation is needed.

The results reported here also show that artificial diets affect the aphids' feeding behaviour and salivary gene expression. Differences were found between the aphids fed on diets (aposymbiotic aphids and control aphids) versus the aphids fed on a plant (normal aphids). The aphids fed on diets spent more time with the stylets outside of the plant (nonprobing, Table 2, variables 2 and 3). These longer nonprobing periods of aphids fed on diets probably had an impact on the overall probing activities, including

activities in the phloem, since the total time of ingestion of phloem sap was shorter (Fig. 2a; Table 2, variable 24). The diet-fed aphids also showed G waveform, which has been correlated with active feeding from xylem and/or mesophyll (Tjallingii 1988; Prado and Tjallingii 1994). This waveform was not found in aphids fed on radish plants (normal aphids) (Table 2, variable 33). Aphids feeding on artificial diets show two main waveform patterns, C and G, by analogy to those described for plants, representing sheath salivation and active ingestion of the diet (Sauvion et al. 2004; Halarewicz and Gabrys 2012). Here, prior to the EPG experiment, aposymbiotic aphids and control aphids spent 4 days ingesting artificial diet, and it is likely that they became adapted to the active ingestion for feeding (similar to drinking from the xylem). Therefore, diet-fed aphids need additional time on the plant to readjust to passive ingestion of sap from phloem tissue. Aphids fed on diets also had longer total and mean duration of potential drops or cell punctures in their way to the phloem (Fig. 2b; Table 2, variables 11 and 12). During these potential drops, small amounts of cell content are ingested by aphids presumably to be "analysed" (Tjallingii and Cherqui 1999), and hence the longer potential drops shown by aphids fed on diets could also be attributed to the changed feeding mode on artificial diets. Similar to EPG results, the expression of two salivary genes, *Mp60* and *Mp61*, showed changes associated with feeding on diets, since aposymbiotic aphids and control aphids had different expression than normal aphids (Fig. 3). The studies that have described salivary proteins secreted by aphids have analysed the saliva secreted into a variety of artificial diets since it is not possible to isolate saliva from the aphids feeding on a host plant (Rao et al. 2013; Harmel et al. 2008; Carolan et al. 2009; Will et al. 2007; Cooper et al. 2010). However, there is little information on how diets affect salivary protein composition. Furthermore, a recent experiment with gelling saliva showed that aphids are able to adapt their salivary secretions in response to different diets (Will et al. 2012). All of the facts discussed above seem to hinder the use of artificial diets in the analysis of the role of salivary proteins in aphid–plant interactions. To further study the role of salivary genes in the plant–aphid interaction and considering the increasing amount of genomic resources that are being generated for aphids (The International Aphid Genomics 2010; Ramsey et al. 2007; Legeai et al. 2010), the use of qRT-PCR likely is a more accurate tool for evaluating the role of candidates salivary genes in response to different plant hosts and diets.

It should be noted that in spite of the fact that the aphids used in the EPG monitoring and in the salivary gene expression experiment belonged to different lineages, the results showed differences related to feeding on artificial diet (diet effect) and differences related to the interruption of *Buchnera* (aposymbiosis effect) in both experiments. Also, although the radish cultivar used in both experiments was different, the same effect was noticed for aposymbiotic aphids in both experiments. Since the responses in the experiments showed the same trend, apparently neither the aphid clone nor the radish cultivar introduced significant changes.

There are also other possible explanations for the results reported here. There is evidence that aposymbiotic aphids have many physiological problems and differences with respect to normal symbiotic aphids, mainly caused by amino acid depletion (Prosser and Douglas 1991, Wilkinson and Douglas 1995, Wang et al. 2010; reviewed in Wilkinson 1998). These differences could cause the delayed host acceptance of aposymbiotic aphids, and so there might be an indirect effect of *B. aphidicola* in the plant–aphid interaction. However, these differences were found comparing aposymbiotic and symbiotic aphids that were treated from the first nymphal stage, and then aposymbiotic aphids did not reach adulthood and had a smaller body size. Here, adult aphids, rather than nymphs, were treated with the antibiotic. Aphids were allowed to complete their development, and only then they were fed with the antibiotic to disrupt *B. aphidicola*. Moreover, all amino acids, including the essential ones, were provided to aphids in the artificial diets in excess quantities to minimize the secondary effects of the aposymbiosis in the aphid physiology. There were no differences in development, size, or weight between aposymbiotic and control aphids (data not shown). Therefore, it should be noticed that the aposymbiotic aphids produced here are not directly comparable to those reported previously in the literature reviewed by Wilkinson (1998).

Another aspect to bear in mind is that in addition to *B. aphidicola*, the aphids can also establish relationships with other types of endosymbiotic bacteria, collectively called secondary symbionts (S-symbionts) or facultative symbionts. The associations are the kind *Buchnera* + S-symbiont. For *M. persicae*, only the presence of *Regiella insecticola* has been reported (Von Burg et al. 2008, Vorburger et al. 2010). Although in the present work, primary symbionts were studied, it is likely that if there are other endosymbionts present in the *M. persicae* clone, they are not involved in the results presented here. To disrupt *R. insecticola*, Vourger et al. (2010) used a different antibiotic, gentamicin rather than rifampicin. Moreover, Koga et al. (2007) report that rifampicin selectively disrupts *B. aphidicola* whilst not having an effect on the secondary endosymbionts of *A. pisum*.

Aphid interactions with *B. aphidicola* have been studied from many different perspectives, including nutritional and metabolic complementation within the aphid body (Douglas 1998; Prosser and Douglas 1992; Douglas et al. 2001;

Viñuelas et al. 2011), associations with the aphids' facultative endosymbionts (Koga et al. 2003; Tsuchida et al. 2002, 2010), and evolution of the *Buchnera* and aphid genome (Shigenobu et al. 2000, 2001; Moran and Degnan 2006; Moran et al. 1993; Nováková et al. 2013). In spite of all of the knowledge gained through these years, relatively little is known about the role of *B. aphidicola* in the aphid–plant interaction. Francis et al. (2006, 2010) showed that *Buchnera* protein amounts change in response to aphid feeding on different hosts. Recently, by in planta overexpression or exogenous application of *Buchnera*'s GroEl, Chaudhary et al. (2014) and Elzinga et al. (2014) demonstrated that the protein is involved in plant–aphid interactions. Here, studying the plant–aphid interaction, it was found that the disruption of *B. aphidicola* from *M. persicae* delays plant acceptance. The proposed explanation is that the aposymbiosis of *B. aphidicola* has a negative effect on the production of proteins or small peptides that act as effectors in the aphid's host plant, and these effectors could be of *Buchnera* and/or aphid origin, but this requires further investigation. Overall, by studying the feeding behaviour of aposymbiotic aphids in comparison with symbiotic aphids, the involvement of *B. aphidicola* in the host plant interaction with *M. persicae* was confirmed.

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