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Culturable Bacteria Associated with the Guts of Pea Aphid, *Acyrthosiphon pisum* (Homoptera:Aphididae)

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Abstract: Gas Chromatography (GC) based on fatty acid profiles and API system (API 20NE and API CH50) techniques were used to identify several bacterial isolates isolated under sterile conditions from the guts of apterous adults and immatures of pea aphid. Pea aphids harboured two bacterial forms in their guts; Cocci Bacterial Cells (CBC) and Rod Bacterial Cells (RBC). Significantly greater density of RBC was obtained in the distal part of the gut than that in the proximal part. Adults and immatures of pea aphid harboured RBC in their guts. The first and second nymphal instars had no CBC. Adults harboured higher densities of CBC and lower densities of RBC than immatures do. Taken as a whole, the guts of apterous adults and immatures of pea aphid contained at least 9 species of cultivable bacteria in five genera. *Stenotrophomonas maltophilia* was found in 31.9% of the samples, was the genus most frequently isolated from the aphid's gut, followed by *Bacillus licheniformis*. *Comamonas acidovorans*, *Pseudomonas putida*, *Pseudomonas* sp., *Micrococcus luteus* and *Micrococcus varians* were absent in the 1st and 2nd nymphal instars.

Key words: Digestive system, bacterial community, gas chromatography, fatty acid profiling, API kit

INTRODUCTION

Invertebrates constitute a much larger group of extant organisms than vertebrates, both in terms of numbers of species and individual organisms, essentially infectable units. Invertebrates have been exposed also to the pathosphere (Burland *et al*., 1998) of bacterial virulence factors for a greater period of evolutionary time than vertebrates, simply because they evolved long before vertebrate lineages (Waterfield *et al*., 2004). Almost all aphids harbor an intracellular symbiotic bacterium, *Buchnera aphidicola* that belongs to the gamma division of the Proteobacteria (Ishikawa, 2001; Koga *et al*., 2003). *Buchnera* is found in specialized cells called bacterocyte or mycetocyte (Ishikawa, 2001). Also aphids often harbour accessory bacteria or secondary endosymbionts found in tissues surrounding the bacteriocytes and in specialized secondary bacteriocytes (Harada *et al*., 1996; Ishikawa, 2001). Recent studies, suggest that accessory bacteria may influence ecologically important traits of aphids, including their performance, plant utilization patterns and resistance to parasitoids (Chen *et al*., 2000; Douglas *et al*., 2006; Fukatsu *et al*., 2001). Most experiments to date have been conducted with the pea aphid *Acyrthosiphon pisum* (Harris), different lineages of which bear none, one, or several of five different bacterial taxa provisionally named: PASS (= R-type), PABS (= T-type) and U-type in γ-Proteobacteria, PAR (= S-type) in α-Proteobacteria and a *Spiroplasma* sp. (Chen *et al*., 2000; Fukatsu *et al*., 2000, 2001; Darby *et al*., 2001). *A. pisum* is also notable for its subdivision into multiple biotypes or races with distinct but overlapping plant ranges (Blackman and Eastop, 2000; Sandstrom and Pettersson, 1994). Research indicated that *Spiroplasma* sp. and PAR could be mildly detrimental to the aphid and that PASS might extend aphid tolerance to high temperatures and promote aphid performance on certain host plant species

(Chen *et al*., 2000; Fukatsu *et al*., 2001; Montllor *et al*., 2002). Under the field conditions, Darby *et al*. (2003) concluded that PABS is not an important factor shaping the performance or plant range of *A. pisum*. Montllor *et al*. (2002) found that in aphids without PASS or PAR, heat stress reduced the number of bacteriocytes to 7% of non-heat-stressed aphids, while aphids with only PASS retained 70% of their bacteriocytes. Bacteriocytes in aphids with PAR but not PASS were reduced to 42% of controls. Although speculative, several horizontal transmission routes are conceivable. Considering its phylogenetic affinity to gut bacteria, the accessory bacteria might sometimes be excreted with honeydew (Fukatsu *et al*., 2000). If oral ingestion can occasionally establish an infection, honeydew, squashed aphids and phloem sap of plants heavily populated by aphids could be sources of infection (Fukatsu *et al*., 2000).

Little research was conducted by Harada's group on identifying the bacteria associated with the guts of adults of Japanese clones of pea aphid and their possible pathogenic effects (Harada and Ishikawa, 1993; Harada *et al*. 1996, 1997). The purpose of this study was to quantify the bacteria associated with the guts of both adults and immatures of pea aphid. Also, to look for culturable forms of these bacteria that are more likely to be present in the guts than in haemolymph and cells (Angela Douglas of University of York, Personal Communication). Then to identify them by using two techniques, API kits and Gas Chromatography based on fatty acid profiling system.

MATERIALS AND METHODS

The Aphid Culture

The experiment was carried out on British clones of pea aphid, *A. pisum* (Harris) derived from single parthenogenetic females collected in the summers of 1998 and 1999 in Berkshire (UK), were maintained on detached one leaf (two leaflets) from pre-flowering *Vicia faba* cv. the Sutton inside Blackman boxes kept under constant temperature room (20±3°C), 60% RH and LD 16:8 h.

Dissection

The guts were dissected from each aphid (apterous adults and immatures) in ice-cold 50 $mmol^{-1}$ Tris-HCl, pH 7.5, with 0.25 mol L^{-1} sucrose and then freed from contaminating haemolymph, embryos, fat bodies and other surrounding tissues with multiple washes in Tris buffer. Each aphid gut was homogenized in 30 μL of ice-cold 50 $mmol^{-1}$ Tris-HCl, pH 7.5, with 0.25 mol L^{-1} sucrose. Thirty six guts were dissected for each adult, 1st, 2nd, 3rd and 4th nymphal instars of the aphid for each test.

Quantification of Bacteria

The gut of each apterous adult was subdivided into two parts that were the proximal part (foregut and stomach) and the distal part of the gut (intestine and rectum). Each aphid gut part was homogenized in 30 μL of ice-cold 50 $mmol^{-1}$ Tris-HCl, pH 7.5, with 0.25 mol L^{-1} sucrose. Ten microliter each homogenate was examined by phase-contrast microscopy at X1000 magnification and the numbers of bacterial cells and ultraspheres in a single field of view were scored (5 views of each homogenate were scored). The number of bacteria per gut was calculated using the following equation:

$$\text{Total No. of bacterial cells} = \frac{\text{No. of bateria counted} \times \text{Volume of homogenate } (\mu L)}{\text{No. of spheres counted} / 39000}$$

The same procedure was used to study the variation of bacterial density among the different aphid stages. Because aphid clones showed significant differences in their weight, the density of bacterial cells were taken per mg aphid weight.

Statistical Analysis

All data were subjected to test of normality using Kolmogorov-Smirnov Test and test of homogenity using Leven's test. Logarithmic transformation was used for data that did not show normal distribution. Means were separated using Least Significant Differences (LSD). Two means were analyzed using t-test at 95% confidence level.

Identification of Bacteria

Thirty six homogenized guts of each stage or nymphal instars were plated separately on microbiological media including nutrient agar (LB agar media) and incubated aerobically at the recommended temperature for each identification test. Three control plates were used in which Tris-buffer was used only without homogenized guts.

Fatty Acid Profiles

Each purified isolated bacterial colony on LB agar nutrient was transferred into a new petridish that contains Soy Tryptic Agar Media (TSA) and incubated under 28°C for 24 h. Bacterial isolates that showed colony growth were identified by Gas Chromatography (GC) system based on fatty acid profiles using the MIDI identification system, by Dr. David Stead (Central Science Laboratories, UK).

Biochemical Tests

When bacterial colony was observed in agar bacterial media, cultures were streaked for purity on the same agar media. Isolates were Gram stained, then exposed to oxidase and catalase tests and a presumptive identification was obtained with the API 20E, API 20NE and API 50CH identification kits (BioMerieux Vitek, Hazelwood, MO).

RESULTS

Culturable bacteria were found in all the dissected guts of immatures and apterous adults of pea aphid (Table 1). The dissected apterous adults contained two bacterial forms in their guts; Cocci Bacterial Cells (CBC) with a diameter of less than 1 μm (91 %) and few of them were of 1 μm and Rods Bacterial Cells (RBC) in which most of them have a length of 2 μm (66%), some of them have a diameter of about 1.5 μm ((22%) and very few RBC have a diameter of 4 μm (2 %). The density of RBC in the guts of the apterous adults was significantly higher than that of CBC (Table 2). Significant differences were observed in the density of bacteria obtained from the different gut sectors (proximal part (foregut and stomach) and distal part (intestine and rectum)) (Table 3). However, significantly more density of RBC was obtained in the distal part of the gut (intestine and rectum) than that in the proximal part (foregut and stomach) (Table 3). CBC were absent in the first and second nymphal instars (Table 1, 4), but the case was different with RBC that were present in the guts of all nymphal instars of pea aphid (Table 1, 5). However, results also showed that adults harbored higher densities of CBC and lower densities of RBC than immatures do (Table 4, 5).

Considerable variation in bacterial genera was observed in different individuals. Bacteria identified are shown in Table 1. A total of 9 different species of bacteria were identified from 5 different genera from a total of 188 isolates (Table 1) from all dissected apterous adults and immatures. Eight isolates could not be identified to the genus. These isolates were isolated from the 3rd nymphal instar (5 isolates), 4th nymphal instars (5 isolates) and apterous adults (4 isolates) (Table 1). Although these isolates were different morphotypes of bacteria, it is not known whether they belong to the same genus (Table 1). The eight unidentified isolates were gram positive cocci, catalase negative and oxidase negative.

Table 1: Cultured bacteria associated with insect guts from the apterous adults and immatures of pea aphid feeding on *Vicia faba* cv. the Sutton

Bacterial isolates	Description	No. of positive samples/32 dissected insect guts					Method of identification
		1st nymphal instar	2nd nymphal instar	3rd nymphal instar	4th nymphal instar	Apterous adults	
Bacillus licheniformis	Gram-positive, catalase-positive, oxidase-negative rods	2	6	5	10	18	FA profiling API 50 CH
Bacillus pumilus	Gram-positive, catalase-positive, oxidase-negative rods	1	2	1	1	4	FA profiling API 50 CH
Bacillus subtilis	Gram-positive, oxidase-positive, catalase-positive bacillus	3	2	2	3	5	FA profiling API 50 CH
Comamonas acidovorans	Gram-negative, oxidase- positive, catalase- positive rods	0	0	4	5	8	FA profiling API 50 CH
Micrococcus luteus	Gram-positive, oxidase-positive, catalase-positive coccus	0	0	4	4	3	FA profiling
Micrococcus varians	Gram-positive, oxidase-negative, catalase-positive coccus	0	0	3	4	2	FA profiling
Pseudomonas putida	Gram-negative, oxidase-positive, catalase-positive rods	0	0	3	5	9	FA profiling
Pseudomonas sp.	Gram-negative, oxidase-positive, catalase-positive rods	0	0	2	1	2	FA profiling
Stenotro-phomonas maltophilia	Gram-negative, oxidase-negative, catalase-positive rod	5	4	8	14	20	FA profiling API 20 NE
Unidentified isolates	Gram-positive, oxidase-negative, catalase-negative cocci	0	0	5	5	3	

Table 2: The density of Cocci Bacterial Cells (CBC) and Rods Bacterial Cells (RBC) in the apterous adults of British clones of pea aphid

Bacterial cell	Log 10 density of bacterial cells mg^{-1} aphid ±SE
CBC	4.82±0.05[b]
RBC	5.10±0.05[a]

Means with the same letter(s) are not significantly different using t-test at 95% confidence level

Table 3: The density of rods bacterial cells in the proximal (foregut and stomach) and distal (intestine and rectum) parts of the gut of apterous adults of British clones of pea aphid

Part of the gut	Log 10 density of bacterial cells mg^{-1} aphid ±SE
Proximal	4.92±0.06[b]
Distal	5.07±0.06[a]

Means with the same letter(s) are not significantly different using t-test at 95% confidence level

Table 4: Density of Cocci Bacterial Cells (CBC) in the 3rd and 4th nymphal instars and apterous adults of British clones of pea aphid

Aphid stage	Density of CBC mg^{-1} aphid	±SE
3rd	45675.23[c]	8703.47
4th	61206.52[b]	10248.43
Apterour adult	79336.74[a]	8534.95

Means with the same letter(s) are not significantly different using Least Significant Differences (LSD) test at 95% confidence level

Table 5: Density of Rod Bacterial Cells (RBC) in the 1st, 2nd, 3rd and 4th nymphal instars and apterous adults of British clones of pea aphid

Aphid stage	Density of RBC mg^{-1} aphid	±SE
1st	576990.11[a]	90153.68
2nd	373991.23[b]	30954.05
3rd	222461.10[c]	22557.22
4th	132210.34[d]	17736.57
Apterour adult	144632.52[d]	16409.22

Means with the same letter(s) are not significantly different using Least Significant Differences (LSD) test at 95% confidence level

There are differences in species composition of the gut microbiota in the different stages of the insect feeding on the same host plant (Table 1). At least 3 genera of bacteria were not cultivated from the 1st and 2nd nymphal instars, these were Comomonas, Micrococcus and Pseudomonas (Table 1). The undetermined bacteria (Table 1) were also not cultivated from the 1st and 2nd nymphal instars. Taking into account that the undetermined genera (Table 1) were gram positive, four of the bacterial genera isolated were gram negative and one genus was gram positive. The predominant genus isolated from the 160 individuals was Stenotrophomonas, present in 31.9% of the samples (Table 1) and it was cultivated from adults and immatures. Three species of Bacillus were cultured from both adults and immatures, these were; *B. licheniformis*, *B. pumilis* and *B. subtilis* (Table 1). However, *B. licheniformis* was the predominant one. Two species of Micrococcus were identified using fatty acid profiling, these were *M. luteus* and *M. varians* and both of them were isolated from 3rd and 4th nymphal instars and adults.

DISCUSSION

The largest class of invertebrates, Insecta, is involved in several types of symbiosis, mainly with bacteria. A high diversity of bacteria has been reported from different types of insects, including gypsy moth, migratory grasshopper, cabbage moth, cotton bollworm (Spiteller *et al*., 2000; Broderick *et al*., 2004; Xiang *et al*., 2006). The bacterial association with insects plays a significant role in the host insect morphogenesis, food digestion, nutrition, antifungal toxin production, pheromone production, regulation of pH, synthesis of vitamins, temperature tolerance, resistance against parasitoid development and detoxification of noxious compounds (Dillon and Dillon, 2004; Genta *et al*., 2006).

Given the importance of these microbial associations to both invertebrates and the environment as a whole, it is remarkable how little is known about them. Relatively few studies have looked at whole microbial communities associated with arthropods. In one such study the collembolan *Folsomia candida* was found to harbour a specific microbial community from which non-indigenous microbes were swiftly removed (Thimm *et al*., 1998). The Folsomia gut flora is diverse, with 26 types of culturable bacteria isolated (Hoffmann *et al*., 1998). Honey bees *Apis mellifera* harbours a range of bacteria, as well as yeasts and moulds in the gut (Gilliam, 1997). In contrast, the grasshopper *Melanoplus sanguinipes* contains an abundant but relatively simple gut microflora consisting mostly of Enterococcus along with Serratia, Pseudomonas and Enterobacter (Mead *et al*., 1988) and there is evidence that related species of Orthoptera have similar microflora (Mead *et al*., 1988; Kaufman *et al*., 2000).

Different lineages of the aphid *A. pisum* showed great variability for the presence or absence of these bacteria, indicating that the relationship is dynamic rather than constant (Sandstrom *et al*., 2001).

Aphid guts do not bear large numbers of microorganism, unlike many animals. This can be attributed to their simple anatomy (e.g., absence of diverticula) and high throughput of phloem sap. However, results of this study showed pea aphid harbored two bacterial forms in their guts; Cocci Bacterial Cells (CBC) and Rod Bacterial Cells (RBC). This indicates that secondary symbionts could have spherical shapes which disagree with Ishikawa (2001) who distinguished between Buchnera (spherical in shape with a diameter of 2.5-4 μm) and the secondary symbionts (rod like or fibrous) according to the shape of cells.

The results presented here confirm that pea aphids can ingest certain culturable bacteria and contain these bacteria in their digestive tracts. The flora in the aphid gut was assumed to have been acquired during probing on the leaf surface, as it is probably the case with most of the bacteria isolated from white flies as well. Srivastava and Rouatt (1963) isolated Sarcina, Micrococcus, Achromobacter and Flavobacterium from aphids. Bacteria have also been reported in the haemocoel of aphids and leafhoppers (Grenier *et al*., 1994; Purcell *et al*., 1986).

In this study, some bacterial isolates were not identified using the gas-liquid chromatography system, but this system provided an identification for most bacterial types including both Gram-negative and Gram positive bacteria. The case is different with API 20NE as most of the supposed non-enteric bacterial isolates were successfully identified using this system. Also, AP 20E failed to identify any isolate in this study.

An agreement in the identification has been obtained between API system and the gas-liquid chromatography system in identifying *Stenotrophomonas maltophilia* that was originally classified as *P. maltophilia* but was transferred to the genus Xanthomonas in 1993 (Palleroni and Bradbury, 1993) and subsequently became the sole member to the genus Stenotrophomonas (Von Graevenitz, 1995), *Comamonas acidovorans* (was known as *Pseudomonas acidovorans*), *Bacillus licheniformis*, *Bacillus pumilus* and *Bacillus subtilis*. *Pseudomonas putida*, *Pseudomonas* sp., *Micrococcus luteus* and *Micrococcus varians* were identified only using gas-liquid chromatography system.

Davidson *et al*. (2000) reported several different types of bacteria that were cultured from surface-sterilized *Bemisia argentifolii*, including *Bacillus* sp., Gram-variable pleomorphic rods and Gram-positive cocci. One of these, *Enterobacter cloacae*, was found within the gut of adult white flies and was mildly pathogenic. Although *E. cloacae* is only mildly pathogenic to *B. argentifolii*, its ability to penetrate whitefly gut cells suggests that this microorganism could be genetically modified to enhance its effectiveness as a biological control agent (Davidson *et al*., 2000). Indiragandhi *et al*. (2007) isolated *Pseudomonas* sp. and *Stenotrophomonas* sp. from the guts of larvae and adults of the diamondback moth.

In other studies, a new bacterial species isolated from pea aphid was described (Harada *et al*., 1997) and showed ability to infect aphid gut and to prevent post-final ecdysis growth of the insect (Harada and Ishikawa, 1997). In the recent study, the cultured bacteria isolated from pea aphid guts are expected to have a pathogenic effect, as *Comamonas acidovorans* (that was isolated from aphid guts) belongs to a group that have few plant pathogens (David Stead of the Central Science Laboratories in UK, Personal communication). Also, the un-identified spherical gram positive bacteria could have pathogenic effects to aphids. Bioassay tests on those bacterial isolates could ensure Wilkinson *et al*. (2001) findings, who offered an evidence to suggest plant mediated interference with nutritional function of symbiotic bacteria in phytophagous insects.

Most insects have a substantial gut microbiota, although there were wide differences among insect taxa and among regions of the gut (Douglas, 2000). Much literature gives misleading estimates of microbial diversity in insect guts, this is attributed to that many or all members of the microbiota are either transient or commensal. In general the density of RBC was greater than that of CBC. This could be ascribed to that some RBC move from the haemocoel to the gut particularly in the distal part of the gut (intestine and rectum) as most RBC concentrated in the distal part of the gut.

Its worthy to indicate that more research is required to identify those bacterial isolates that were not identified by both API and Gas Chromatography techniques. This could be done by using molecular techniques.

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