Bacteria Associated with some *Dacus* Species (Diptera : Tephritidae) and their Host Fruit in Queensland

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Abstract

Over a period of 18 months the bacteria associated with approximately 70 adult flies of four *Dacus* species were isolated and identified. The flies were *D. tryoni* (Froggatt) and *D. neohumeralis* Hardy from guava (*Psidium guajava* L.), mulberry (*Morus nigra* L.) and peach [*Prunus persica* (L.) Batsch], *D. cacuminatus* (Héring) from wild tobacco (*Solanum mauritianum* Scop.), and *D. musae* (Tryon) from banana (*Musa paradisiaca* L.), and were collected in the field when these host plants were fruiting. All flies examined were surface-sterilized prior to aseptic dissection in which crop and mid-gut (stomach) or oesophageal bulbs were removed for culturing. Bacteria were also isolated from faeces of field-collected flies, as well as from host fruit surfaces, oviposition sites and larvae-infested tissue in host fruit. The predominant bacteria found in the alimentary tract of flies and in associated fruit specimens were members of the family Enterobacteriaceae. *Klebsiella oxytoca*, *Erwinia herbicola* and *Enterobacter cloacae* were the most frequently isolated species, with *Serratia* spp., *Citrobacter freundii*, *Proteus* spp., *Providencia rettgeri* and *Escherichia coli*, being found less frequently. No one bacterial species was found to be consistently associated with any one fly species. The bacterial species found most frequently in the alimentary tract were also found in large numbers on the surfaces of host fruit and in stung fruit.

Introduction

In the family Tephritidae, a close association between the insect and its alimentary tract microflora has long been assumed (Petri 1910). The bacteria of the alimentary tract have been reported to be obligate symbionts (Petri 1910; Hagen 1966; Baerwald and Boush 1968; Lambrou and Tzanakakis 1978; Miyazaki *et al.* 1983); non-symbionts (Yamvrias *et al.* 1968; Tsiropoulos 1983; Rossiter *et al.* 1983; Fitt and O'Brien 1985; Howard *et al.* 1985); or ingested food (Dean and Chapman 1973; Drew *et al.* 1983). The importance of the alimentary tract bacteria in the biology of fruit flies has thus been generally accepted, but the precise nutritional role these bacteria play in the life of the larva or the adult fly remains poorly defined.

Many different microbiological methods have been employed to determine the bacteria associated with fruit flies (Allen *et al.* 1934; Rubio and McFadden 1966; Boush *et al.* 1972; Tsiropoulos 1976). Only limited attempts have been made to study the microflora in different parts of the alimentary tract (Dean and Chapman 1973). If plant-surface microorganisms provide a natural food source as suggested by Courtice and Drew (1984) the leaf and fruit microflora of host, and possibly non-host, trees may be a vital component in the fruit fly ecological system.

In attempting to understand further the role which bacteria may play in the biology and ecology of fruit flies in Queensland, the present investigations were aimed at

a comprehensive survey of bacteria associated with different species of adult *Dacus* spp. collected in the field from various host trees at different times of the year. The predominant microflora of various parts of the alimentary tract (as described by Drew *et al.* 1983), together with the types of organisms present on the surfaces of host fruit and in oviposition sites and larval-infested tissue of stung fruit, were also investigated.

Material and Methods

Field Collection of Flies and Fruit

Adults of four fruit fly species were collected singly in sterile glass tubes from their respective hosts, *Dacus cacuminatus* (Héring) from wild tobacco (*Solanum mauritianum* Scop.), *Dacus tryoni* (Froggatt) and *Dacus neohumeralis* Hardy from guava (*Psidium guajava* L.), mulberry (*Morus nigra* L.), and peach [*Prunus persica* (L.) Batsch] and *Dacus musae* (Tryon) from banana (*Musa paradisiaca* L.), when these host plants were fruiting.

Flies collected in suburban areas of Brisbane were held at room temperature prior to examination. All flies, with the exception of *D. musae* specimens, were examined within 2 h of collection. *D. musae* specimens were collected from north Queensland, frozen in liquid nitrogen and transported to the Brisbane laboratory for examination. Laboratory-reared *D. tryoni* flies were obtained from a colony which had been maintained under laboratory conditions for many years.

Fruit from host trees from which flies were obtained were placed singly in sterile containers at the same time as the flies were collected.

Dissection of Flies and Isolation of Microorganisms from the Alimentary Tract

Prior to examination, all live flies were anaesthetized by cold (3 min at $-4°C$), the anal and mouth openings sealed with sterile wax and the whole fly surface-sterilized by washing in 70% (v/v) alcohol (30 s), 0.25% (w/v) sodium hypochlorite (1 min), and sterile distilled water (30 s). The flies were then mounted on sterile wax blocks (dorsum downwards) and dissected aseptically under sterile water. Crop and midgut content colour, size of crop and state of ovarian development in females were recorded. Various parts of the alimentary tract as described by Drew *et al.* (1983), viz. crop, mid-gut (stomach) and oesophageal bulb (diverticulum), were carefully removed and spread directly onto peptone–yeast extract–agar (PYEA; peptone 10 g, yeast extract 5 g, NaCl 5 g, agar 15 g, distilled water 1 litre, pH adjusted to 7.2). To reduce the risk of cross-contamination during dissection, oesophageal bulbs were only removed from flies in which the remainder of the alimentary tract was left intact. Faecal deposits, if present in the collection tubes by the time of dissection, were suspended in a drop of sterile water and spread onto PYEA plates.

All plates were incubated aerobically at 30°C and examined daily. A qualitative assessment of the number and types of colonies present on each plate was made after 48 h and representative colonies of the predominant types on each plate were subcultured, purified and maintained on PYEA slopes at 4°C. Plates with less than five colonies were recorded as 'insignificant bacterial growth' and no subcultures were made from these specimens.

Isolation of Bacteria from Fruit Surfaces

Since a comprehensive survey of host fruit surface organisms was beyond the scope of these studies, examination of the microflora of host fruit near which flies had been collected was aimed primarily at determining the presence or absence of bacteria commonly found in flies (hence referred to as 'fruit-fly type bacteria'). Each fruit was washed in sterile distilled water for 1 min with gentle hand agitation. Aliquots (0.1 ml) of wash solution were plated on PYEA and after 2 days representative colonies of 'fruit-fly type' bacteria were subcultured for identification.

Isolation of Bacteria from Fruit Stung by Fruit Fly

Field-collected stung fruit examined were guava, wild tobacco, mulberry, peach, and banana. Fruit which contained visible oviposition sites was washed and surface-sterilized by dipping for 1 min in running tap water, 2 min in 70% (v/v) alcohol, 5 min in 0.25% (w/v) sodium hypochlorite and 1 min in sterile distilled water. Microorganisms present in oviposition sites were isolated by aseptically dissecting a small wedge of tissue through the centre of the site and smearing the cut edge over the surface

of a PYEA plate. If larvae were present in the fruit, a loopful of the associated rotting tissue was similarly plated.

Identification of Bacteria

All bacterial isolates were initially Gram-stained and tested for oxidase activity (Oxidase Reagent Droppers, Marion Scientific, Kansas City, Missouri). Almost all isolates from flies, oviposition sites and larval rot were Gram-negative, oxidase-negative, rod-shaped organisms which were identified by the API–20E system for Enterobacteriaceae. The few organisms isolated which did not belong to this family were not further characterized. Motility was determined by growing organisms in semi-solid motility medium (peptone 10 g, beef extract 3 g, NaCl 5 g, agar 4 g, distilled water 1 litre) followed by microscopic examination of hanging-drop preparations.

Results

Microflora of Field-collected Flies

Bacteria were isolated from four *Dacus* species collected from host plants during months of peak fruiting (Table 1). The numbers of specimens examined, bacterial

Table 1. Field collection of *Dacus* species for microbiological examination

Fly species	Host tree	Time of collection	No. of flies examined
D. cacuminatus	Wild tobacco	February–April	26
D. tryoni	Guava	April	11
D. neohumeralis	Guava	April	1
D. neohumeralis	Mulberry	October	7
D. neohumeralis	Peach	November	4
D. tryoni	Peach	November	10
D. musae	Banana	March	14

isolates obtained and percentage of these isolates identified as Enterobacteriaceae are shown in Table 2.

Table 2. Bacteriological examination of the alimentary canal, faeces and host fruit tissue of field-collected *Dacus* species

Material examined	No. of specimens examined	No. of specimens containing bacteria[A]	No. of bacterial isolates[B]	Isolates identified as Enterobacteriaceae	
				No.	%
Insect					
Crop	52	34	38	30	79
Mid-gut	53	52	65	59	91
Oesophageal bulb	16	12	12	11	92
Faeces	30	20	20	20	100
Fruit					
Oviposition site	19	19	23	21	91
Larval rot	17	17	19	14	74

[A] More than five colonies isolated on PYEA.

[B] One or more bacteria were isolated from each plate on the basis of different colony characteristics.

Although no accurate quantitative assessments of bacterial populations were made in this investigation, some general conclusions could be drawn concerning the numbers of bacteria present in various sections of the alimentary tract. With the exception

of one newly emerged fly, all specimens examined contained very large numbers of bacteria in the mid-gut. However, bacterial populations of crops, bulbs and faeces were more variable, and 35% of crops, 25% of bulbs and 33% of faeces examined contained very low numbers of bacteria (i.e. less than five colonies per plate). Crops which appeared empty (deflated) on dissection consistently contained very low numbers of bacteria, but large fluid-filled crops did not always contain large numbers of bacteria.

When primary isolation plates were examined after 48 h, usually no more than two colony types were detected from any one specimen, and frequently only one colony type appeared to be present.

Of 135 isolates obtained from the alimentary tract and faeces of field-collected flies, 120 were oxidase-negative, Gram-negative rods which were successfully identified as species in the family Enterobacteriaceae by the API−20E system. Of the 15 remaining unidentified isolates, a small number were oxidase-positive *Pseudomonas* species. Only four of the 73 field flies examined were found to contain microorganisms other than Gram-negative rods. They were two flies which contained Gram-positive cocci and two flies which contained small Gram-positive rods as well as the commonly found species in the Enterobacteriaceae. Yeasts were not detected in any part of the alimentary canal. The lower percentage of Enterobacteriaceae in crops (79% compared with 91 and 92% in mid-guts and bulbs, respectively) indicated the more varied microflora of this part of the alimentary tract (Table 2).

Table 3. Isolation of Enterobacteriaceae species from field-collected *Dacus* species and from stung host fruit

Bacterial species	No. of isolations from					
	Crop	Mid-gut	Bulb	Faeces	Oviposition site (fruit)	Larval rot (fruit)
Erwinia herbicola	6	6	1	7	4	3
Klebsiella oxytoca	7	17	1	1	5	3
Enterobacter cloacae	8	13	2	9	11	7
Citrobacter freundii	4	10	4	1	0	0
Proteus and *Providencia* spp.	4	7	2	1	1	1
Escherichia coli	1	4	0	1	0	0
Serratia spp.	0	2	1	0	1	0

The species of Enterobacteriaceae found associated with field flies and with oviposition sites and larval rot in infested host fruit are shown in Table 3. *Erwinia herbicola* (syn. *Enterobacter agglomerans*), *Klebsiella oxytoca*, and *Enterobacter cloacae* were the most frequently isolated species, with *Citrobacter freundii*, *Proteus mirabilis* and *P. vulgaris*, *Providencia rettgeri*, *Escherichia coli*, *Serratia liquefaciens* and non-pigmented *S. marcescens* being less frequently isolated.

Fly species, sex, host tree and time of collection did not appear to affect the bacterial species present in the alimentary tract.

No one species of bacterium was consistently associated with any one species of fly. *E. herbicola*, *K. oxytoca* and *E. cloacae* were isolated in approximately equal numbers from crops. *K. oxytoca* was the species most commonly found in the mid-gut and *E. cloacae* the most common isolate from faeces samples. The number of oesophageal bulbs examined was much smaller than the number of crops and mid-guts, but in the bulbs which did contain moderate numbers of bacteria, *C. freundii*

was the most commmon bacterium isolated. Five of the 13 *E. herbicola* strains isolated from the alimentary tract of flies were yellow-pigmented.

Microorganisms Associated with Oviposition Sites and Larval Rot

Bacteriological examination of oviposition sites and larval-infested tissue in host fruit showed large numbers of bacteria present in all specimens from a variety of fruit (Table 2). Provided stung fruit were adequately surface-sterilized, each oviposition site or area of larval rot yielded bacteria of only one or two colony types. In all, 91% of the isolates from oviposition sites and 74% of isolates from larval rot were identified as Enterobacteriaceae. The three most common species found in the alimentary tract of adult field flies, viz. *E. herbicola*, *K. oxytoca* and *E. cloacae*, were also the three most commonly isolated from inside host fruit, with *E. cloacae* occurring twice as frequently as the other two species.

C. freundii and *E. coli* were found in the alimentary tract of flies but have not been isolated from field-stung fruit, while non-pigmented *Serratia* species have been isolated from only one oviposition site and not from larval-infested tissue.

When isolations were made from firm tissues of guava fruit up to 10 mm from unhatched eggs, the same bacteria as in the oviposition site were found in the apparently intact adjacent fruit tissue. This indicated that the bacteria can spread in host tissue ahead of the larvae. Fruit tissue well removed from an oviposition site was sterile. When larvae were present and tissue breakdown was more advanced a lower proportion (74%) of the bacteria isolated from host tissue belonged to the Enterobacteriaceae (Table 2). This may indicate secondary invasion of infested host fruit tissue by resident or casual fruit-surface organisms.

Table 4. Bacteria isolated from field-collected *Dacus* species and from the surface and interior of host fruit from trees in which flies were collected

Bacterium	Flies	Host tree source[A] Fruit surface	Oviposition site and/or larval rot
Erwinia herbicola	TGMP	TGPB	M
Klebsiella oxytoca	TGMPB	PB	MPB
Enterobacter cloacae	TGMPB	TGP	GMPB
Citrobacter sp.	TGMPB	G	
Proteus spp. and Providencia rettgeri	TMPB	P	GP
Serratia spp.	GB	GB	G

[A] T, wild tobacco; G, guava; M, mulberry; P, peach; B, banana.

Bacteria Present on Host Fruit Surfaces

Bacteria found in the alimentary tract of field-collected flies were usually found on the surfaces of host fruit from trees in which flies had been collected (Table 4). A comparison of bacteria on caged (protected from fruit flies) and uncaged (accessible to fruit flies) guava fruit in the field has shown that immature fruit in both cases supported relatively few Enterobacteriaceae on their surfaces. As fruit matured, and flies began frequenting the uncaged tree, the proportion of 'fruit-fly type' bacteria on exposed fruit increased greatly (Drew and Lloyd 1986).

Bacteria Present in Laboratory-reared D. tryoni

During the course of this investigation the bacteria present in the alimentary canal of laboratory-reared *D. tryoni* were also identified. Results showed that Enterobacteriaceae were the predominant organisms present in crops and mid-guts but, unlike field-collected flies, laboratory flies contained predominantly pigmented and non-pigmented *Serratia marcescens* and *S. liquefaciens*. It has also been shown that *Drosophila melanogaster* (Meigen) frequently found around laboratory-reared colonies, carry large numbers of *S. marcescens* and are consequently a likely source of reinfection of newly emerged laboratory-reared flies.

Discussion

Results presented here show that the natural alimentary tract microflora of four field-collected *Dacus* species consisted predominantly of seven species of bacteria in the family Enterobacteriaceae (Table 3). *K. oxytoca*, the predominant species in the midgut, and *E. cloacae*, the predominant species in faeces, were also the most common organisms found in the alimentary tract of *Rhagoletis pomonella* (Dean and Chapman 1973; Rossiter *et al.* 1983), and *K. oxytoca* was the most common isolate from oesophageal bulbs of the same species (Howard *et al.* 1985). The fact that one-third of the faeces samples cultured contained no bacteria is consistent with the earlier findings of Drew *et al.* (1983). One-quarter of the oesophageal bulbs dissected from field flies contained no bacteria recoverable on PYEA. Hence the importance of this organ in maintaining a constant supply of bacteria to the gut, as suggested by Ratner and Stoffolano (1982, 1984) for *R. pomonella*, is not clear from the data for the *Dacus* species studied here.

The bacteriological methods adopted here, as in most published work on bacteria associated with fruit flies, were limited to some extent in that they were designed to detect rapidly growing aerobic organisms with no special growth requirements. As no one species of the bacteria detected was consistently associated with any one species of fly, the term 'symbiosis' implying a specific, mutualistic relationship does not seem appropriate (Brooks 1963). The bacteria belonging to the Enterobacteriaceae which have been characterized here are probably best considered as the predominant commensal inhabitants of the alimentary tract, at least until their nutritional role is better-defined. Other recent studies on the bacteria associated with fruit flies have also questioned the existence of an obligate symbiotic relationship (Yamvrias *et al.* 1970; Tsiropoulos 1983; Fitt and O'Brien 1985; Howard *et al.* 1985). The possibility exists, however, that the fruit fly alimentary tract may contain small numbers of other organisms, possibly slow growing or with more fastidious growth requirements, which are as yet undetected. Such organisms, if present, could be involved in an obligate symbiotic relationship with the fly.

The importance of studying field-collected flies has been demonstrated by results presented here and elsewhere (Tsiropoulos 1983) in that marked differences are shown between the bacteria found in wild and laboratory-reared flies. Fitt and O'Brien (1985) found *Serratia* species to be the most common in their 'wild' flies which were reared in the laboratory from field-collected, larval-infested fruit. Our studies have shown that *Serratia* species were common in the alimentary canal of adult laboratory flies but were not common in field-collected adult flies. It appears that the predominance of *Serratia* species, in particular red-pigmented *S. marcescens*, in adult flies may be a laboratory-acquired characteristic related to the presence of

this organism in *D. melanogaster* which commonly infests laboratory-reared colonies in Queensland.

The alimentary tract bacteria were also consistently associated with host fruit surfaces, oviposition sites and larval rot in stung fruit (Table 3). *E. cloacae* was the most common isolate in infested host fruit and Fitt and O'Brien (1985) have shown that this organism has a beneficial effect on development of *D. jarvisi* larvae in artificial media. Fitt and O'Brien (1985) also examined the bacteria in larval-infested tissue of various host fruits of *Dacus* species and found several species (*E. cloacae, K. pneumoniae, E. herbicola* and *S. liquefaciens*) to be present.

The means by which bacteria are introduced into stung fruit at oviposition is as yet unclear. Although Petri (1910) claimed that there was an elaborate anatomical arrangement in the ovipositor of *D. oleae* for smearing bacteria on to the surfaces of eggs as they were laid, no such organs are known in tropical *Dacus* species. It has been suggested by Courtice and Drew (1984) that female flies may inoculate the surface of fruit with regurgitated spittle prior to ovipositing. Preliminary investigations have shown that the typical alimentary canal bacteria are present in fly spittle. It is thus likely that these bacteria are spread on the surfaces of host fruit and then introduced into the fruit by the ovipositor.

Drew *et al.* (1983) have shown that fruit flies can be reared on cultures of their own alimentary tract bacteria as a nitrogenous source. Drew and Lloyd (1986) have shown that there is a marked increase in the population of these 'fruit-fly type' bacteria on fruit surfaces, once flies begin frequenting a host tree. Hence, it is possible that, once these alimentary tract bacteria have been introduced onto a fruit surface, they may proliferate there, and in so doing, provide a natural source of protein food for foraging adult flies.

The predominant microflora of Queensland fruit flies having been identified, further studies are now in progress to define the possible multiple functions of these bacteria in the life of this insect.

Acknowledgments

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