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Bacteria belonging to the genus *Bacillus* associated with three species of solitary bees *

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Summary — Brood provisions from *Centris flavofasciata* and *Xylocopa californica arizonensis*, larval feces and pollen collected by *X c arizonensis*, and the alimentary canal of adult female *Crawfordapis luctuosa* were examined for micro-organisms, particularly spore-forming bacteria belonging to the genus *Bacillus*. *Bacillus* species were associated with some samples from all 3 bees. Brood provisions of *C flavofasciata* contained *B licheniformis*. *Bacillus cereus*, *B subtilis*, and an unidentified *Bacillus* similar to *B sphaericus* were isolated from the gut of *Crawfordapis*. Pollen collected by *X c arizonensis* contained *B circulans*, *B pumilus*, and *B subtilis*. The latter species was also associated with provisions of a young carpenter bee larva. These results and our previous findings on microflora of *Anthophora* sp, *Apis mellifera*, *Centris pallida*, *Melipona fasciata*, and a necrophage of the genus *Trigona* demonstrate that *Bacillus* species are common associates of Apoidea and could participate both in metabolic conversion of food and in the control of competing and/or spoilage micro-organisms.

Apoidea / micro-organism / *Bacillus*

From our previous results which demonstrated the association of spore-forming bacteria belonging to the genus *Bacillus* with the stored food of both social and solitary species of Apoidea, we postulated that female bees may inoculate food with *Bacillus* species which could pre-digest, convert, and/or preserve stored food (Gilliam, 1979; Gilliam *et al*, 1984, 1985, 1990). Since *Bacillus* species are recognized and utilized for their ability to produce and secrete numerous enzymes, antibiotics, and fatty acids, they could play a role both in the metabolic conversion of food of bees and in the control of competing or spoilage microbes.

Associations of *Bacillus* species with pollen collected and stored by honey bees, *Apis mellifera* (Gilliam, 1979), and by a stingless bee, *Melipona quadrifasciata*

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(Machado, 1971), have been reported. Machado (1971) also found at least 1 species of *Bacillus* in the larval food of 13 stingless bee species. Gilliam *et al* (1984, 1985) examined larval provisions of *Anthophora* sp, *Centris pallida*, and an obligate necrophage of the genus *Trigona* and found that all contained 1–5 species of *Bacillus* but no other micro-organisms. The same species of *Bacillus* (*B circulans*, *B licheniformis*, *B megaterium*, *B pumilus*, and *B subtilis*) were found in glandular cell provisions made by the *Trigona* in the tropical wet forest and in pollen collected and stored as bee bread by *A mellifera* in the Arizona desert. *Bacillus circulans* was isolated from larval provisions of the solitary, soil-nesting bees, *Anthophora* sp and *C pallida*; *B coagulans*, *B firmus*, and *B megaterium* were also present in provisions of *C pallida*. Recently, we reported *B alvei*, *B circulans*, and *B megaterium* from stored food of a social stingless bee, *Melipona fasciata*, from Panamá (Gilliam *et al*, 1990). *Bacillus megaterium* was isolated from brood provisions, pollen from storage pots, and honey from storage pots. The 2 other species of *Bacillus* were found in a few honey and brood provision samples but not in stored pollen. Few other micro-organisms were found.

In this paper, we report *Bacillus* species associated with 3 species of solitary bees. Unfortunately, solitary bees and their provisions are not readily obtainable. Nesting sites are difficult to locate, and bees and provisions are present only for a few days or weeks within the year. *Centris flavofasciata* samples were collected in Guanacaste Province, Costa Rica in February 1986. *Crawfordapis luctuosa* adult females were obtained in October 1983 from a nesting aggregation described by Roubik and Michener (1985) in Chiriquí Province, Panamá. Samples from the carpenter bee, *Xylocopa californica arizonensis*, from Box Canyon in the Santa Rita Mountains in southern Arizona were collected in May 1986. The first 2 bee species nest in soil; *X c arizonensis* nests in the previous season's infructescences of *Agave*, *Yucca*, and *Dasylirion* (Agavaceae). *Centris* and *Xylocopa* are anthophorids, and *Crawfordapis* is a colletid bee.

Bees and cells were frozen at – 20 °C and/or freeze-dried until samples (table I) were removed for microbiological analyses for bacteria, molds, and yeasts by procedures previously described (Gilliam *et al*, 1985, 1990). Adult *Crawfordapis* female bees were surface-sterilized with ethanol, rinsed in 3 washes of sterile distilled water, and dissected under sterile conditions to remove the alimentary tract which was divided into 2 sections, esophagus plus crop and ventriculus through rectum, each of which was individually homogenized in 1 ml of sterile distilled water and then plated. Pollen and fecal pellets from *Xylocopa* were also homogenized before plating. The pollen sample had been collected by the female, mixed with nectar, and stored outside the cells against the burrow wall close to the entrance hole. Brood provisions from individual cells were plated directly. Nutrient agar, TYG (tryptic soy agar with glucose and yeast extract), Czapek solution agar, YM-1 agar, and thioglycollate medium incubated at 25 °C and 37 °C were the isolation media. Thioglycollate medium was used to test for the presence of microaerophilic and anaerobic organisms, and any growth in these tubes was transferred to nutrient agar and TYG plates. When growth on these media was poor, plates of brain heart infusion agar were also inoculated. Plates were incubated aerobically, under 5% CO_2, and anaerobically to determine whether the organisms were facultative, microaerophilic, or anaerobic.

Table I. Micro-organisms associated with 3 solitary bees. [a] EC = esophagus and crop; [b] VR = ventriculus through rectum; [c] ND = not determined.

Bee	*Sample*	*Micro-organism*	*Number of isolations*
Centris flavofasciata	provision 1 (egg)	*Bacillus licheniformis*	3
	provision 2 (larva)	*B licheniformis*	2
		orange actinomycete	1
		white mold	1
Crawfordapis luctuosa	Female 1		
	EC[a]	ND[c]	
	VR[b]	*B subtilis*	9
	Female 2		
	EC	none	
	VR	none	
	Female 3		
	EC	Gram-positive anaerobic bacterium	2
	VR	none	
	Female 4		
	EC	none	1
	VR	*B cereus*	
	Female 5		
	EC	none	
	VR	unidentified *Bacillus* sp	2
Xylocopa californica arizonensis	provision 1		
	(large larva)	mucoid bacterium	8
		white mold	5
		brown mold	1
	feces	bacterium	1
	provision 2		
	(large larva)	small yeast	5
	feces	mold	1
	provision 3		
	(large larva)	mold	2
		small yeast	1
	feces	none	
	provision 4		
	(very young larva)	*Alternaria* sp	3
		B subtilis	2
		Stachybotrys sp	1
	provision 5		
	(late first instar larva)	small yeast	15
		mold	1
	feces from larvae 6	none	
	pollen	small yeast	9
		gray mold	4
		B pumilus	2
		green mold	2
		white mold	2
		B circulans	1
		B subtilis	1
		Streptomyces sp	1

Bacillus species were associated with some of the samples from each of the 3 bee species tested (table I). Brood provisions of *C flavofasciata* contained *B licheniformis*. *Bacillus cereus*, *B subtilis*, and an unidentified *Bacillus*, which gave reactions in taxonomic tests that were closest to those for *B sphaericus*, were isolated from the gut but not the crop of *Crawfordapis* adult females bees. Pollen collected by *X c arizonensis* contained *B circulans*, *B pumilus*, and *B subtilis*. The latter species was also isolated from provisions containing a young carpenter bee larva.

Pollen (74% *Prosopis*, 22% *Fouquieria*, 4% *Argemone*, 0.4% Liliaceae, 0.4% miscellaneous legumes) collected and stored by *X c arizonensis* contained the same *Bacillus* species as almond, *Prunus dulcis*, pollen collected and stored by *A mellifera* with the exception of *B megaterium* (Gilliam, 1979). *Bacillus licheniformis* and *B subtilis*, species isolated from brood provisions in the present experiment, were also found previously in brood provisions of the necrophage of the genus *Trigona* (Gilliam *et al*, 1985). *Bacillus cereus* and *B subtilis*, isolated from *Crawfordapis* ventriculus-rectum, have been reported from the alimentary canal of *A mellifera* workers (Gilliam and Valentine, 1976; Gilliam and Morton, 1978) and queens (Gilliam, 1978).

Since the aim of our study was to determine associations of *Bacillus* species and bees, detailed efforts were not made to identify other micro-organisms that were isolated. From the limited samples available for analyses, it appears that diverse microbial associations as found previously for *A mellifera* (Gilliam, 1989) may occur with the carpenter bee, *X c arizonensis*, since bacteria, yeasts, and molds were isolated.

Since 3 of 5 female *Crawfordapis* contained *Bacillus* species in the gut, examination of additional bees and brood provisions, which were unavailable when our collections were made, are necessary to determine whether *Bacillus* species are responsible for the absence of other microbes in the gut and whether they are transferred to provisions. Microbiological data from *C pallida* (Gilliam *et al*, 1984) and *C flavofasciata* show the association of *Bacillus*, although of different species, with *Centris*.

As a screening process to assess potential enzymatic contributions by the *Bacillus* species isolated, strains were tested for production of 19 enzymes with the API ZYM system as previously described (Gilliam *et al*, 1985). Ten of the 19 enzymes were produced by strains associated with each of the 3 bees, although the specific enzymes varied (table II). All strains produced caprylate esterase-lipase. Enzymes produced in the highest concentrations were as follows: acid phosphatase (≥ 40 nanomoles) by *B cereus* and *B subtilis* from *Crawfordapis*, chymotrypsin (≥ 30 nanomoles) by *B circulans* from pollen collected by *Xylocopa* and by *B subtilis* from *Crawfordapis*, alkaline phosphatase (≥ 30 nanomoles) by *B pumilus* from pollen collected by *Xylocopa*, caprylate esterase-lipase (≥ 30 nanomoles) by *B pumilus* and *B subtilis* from pollen collected by *Xylocopa*, and leucine aminopeptidase (≥ 30 nanomoles) by *B subtilis* from *Crawfordapis*.

Results from taxonomic tests showed that all isolates produced catalase. All isolates from *C flavofasciata* and *Xylocopa* produced proteases that hydrolyzed casein; all from *Xylocopa* fermented glucose with acid production and produced proteases that liquefied gelatin; all from *Centris* produced amylase, reduced nitrates to nitrites, and fermented glucose, arabinose, mannitol, and trehalose with acid production; and all from *Centris* and *Crawfordapis* and most from *Xylocopa* grew in acid pH

Table II. Summary of enzymes produced by *Bacillus* species associated with 3 species of solitary bees. [a] V = variable.

Enzyme	Centris flavofasciata	Crawfordapis luctuosa	Xylocopa californica arizonensis
Alkaline phosphatase	+	+	V
Butyrate esterase	V[a]	–	V
Caprylate esterase-lipase	+	+	+
Myristate lipase	–	–	–
Leucine aminopeptidase	+	V	+
Valine aminopeptidase	–	V	V
Cystine aminopeptidase	–	–	–
Trypsin	–	V	–
Chymotripsin	V	+	V
Acid phosphatase	V	+	V
Phosphamidase	V	+	V
α-Galactosidase	–	–	–
β-Galactosidase	+	–	–
β-Glucuronidase	–	–	–
α-Glucosidase	+	V	V
β-Glucosidase	V	V	V
N-Acetyl-β-glucosaminidase	–	–	–
α-Mannosidase	–	–	–
α-Fucosidase	–	–	–

and at high osmotic pressure. Otherwise, there was variability in these characteristics with the exception of the failure of isolates from *Crawfordapis* to ferment arabinose, mannitol, and xylose.

Therefore, based on results from taxonomic tests and the API ZYM system, isolates from provisions of *Centris* were the most active with regard to carbohydrate catabolism, and those from the ventriculus-rectum of *Crawfordapis* were the least active. Isolates from all sources produced enzymes involved in protein and lipid catabolism. These data provide information on the biochemical capabilities of the bacteria, but further work is necessary to ascertain how they relate to the metabolism and biochemistry of the bees.

Our work on the association of *Bacillus* species and bees is continuing as more material becomes available. After these surveys are completed, more detailed research will be required to assess the dynamics, ecology, and specific contributions of these micro-organisms to bees.

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Résumé — Bactéries appartenant au genre *Bacillus* associées à trois es-

pèces d'abeilles solitaires. Afin de poursuivre l'inventaire des bactéries sporulantes à Gram positif du genre *Bacillus*, nous avons mené une étude microbiologique des provisions à couvain de *Centris flavofasciata* et de *Xylocopa californica arizonensis*, du tube digestif de la femelle adulte de *Crawfordapis luctuosa*, des fécès de larves de *X c arizonensis* et du pollen collecté par celle-ci. Des espèces de *Bacillus* ont été trouvées dans certains échantillons de ces 3 abeilles. Les provisions à couvain de *C flavofasciata* contenaient *B licheniformis*. *B cereus*, *B subtilis* et un *Bacillus* non identifié, semblable à *B sphaericus*, ont été isolés de l'intestin de *Crawfordapis*. Le pollen récolté par *X c arizonensis* contenait *B circulans*, *B pumilus* et *B subtilis*. Cette dernière espèce était aussi associée aux provisions d'une jeune larve de xylocope.

Ces résultats sont comparés avec les précédents concernant la microflore *d'Anthophora sp*, *Apis mellifica*, *Centris pallida*, *Melipona fasciata* et une abeille nécrophage du genre *Trigona*. Ils montrent que les espèces de *Bacillus* sont communément associées aux Apoïdes. Ces bactéries pourraient prendre part, à la fois à la transformation métabolique de la nourriture des abeilles et à la lutte contre les micro-organismes concurrents qui gâtent la nourriture.

Apoidea / microorganisme / *Bacillus*

Zusammenfassung — Bakterien aus der Gattung *Bacillus*, die bei drei Arten Solitärer Bienen vorkommen. Um zusätzliche Bienenarten auf das Vorkommen Gram-positiver, sporenbildender Bakterien aus der Gattung *Bacillus* zu überprüfen, wurden mikrobiologische Untersuchungen durchgeführt an 1) Brut-Nahrungsvorräten von *Centris flavofasciata*; 2) dem Verdauungstrakt adulter Weibchen von *Crawfordapis luctuosa*; 3) Brut-Nahrungsvorräten, Larven-Fäzes und Pollen aus Nestern von *Xylocopa californica arizonensis*. Bei allen diesen drei Bienen wurden in einigen Proben *Bacillus*-Arten gefunden. Die Brutnahrung von *C flavofasciata* enthielt *B licheniformis*. *Bacillus cereus*, *B subtilis* und ein unbestimmter *Bacillus*, ähnlich dem *B sphaericus*, wurden aus dem Darm von *Crawfordapis* isoliert. Der von *X c arizonica* gesammelte Pollen enthielt *B circulans*, *B pumilus* und *B subtilis*. Letzere Art kam auch in den Vorräten einer jungen Larve der Holzbiene vor. Diese Resultate werden mit unseren früheren Ergebnissen über die Mikroflora von *Anthophora*, *Apis mellifera*, *Centris pallida*, *Melipona fasciata* und einem Nekrophagen (Aasfresser) der Gattung *Trigona* verglichen. Sie zeigen, daß *Bacillus*-Arten bei den Apoidea ganz allgemein vorkommen. Diese Bakterien könnten sowohl an der metabolischen Umwandlung der Bienennahrung wie an der Kontrolle der konkurrierenden, die Nahrung verderbenden Mikroorganismen beteiligt sein.

Apoidea / Mikroorganismus / *Bacillus*

REFERENCES

Gilliam M (1978) Bacteria belonging to the genus *Bacillus* isolated from selected organs of queen honey bees, *Apis mellifera*. *J Invertebr Pathol* 31, 389-391

Gilliam M (1979) Microbiology of pollen and bee bread: the genus *Bacillus*. *Apidologie* 10, 269-274

Gilliam M (1989) Microbial ecology of honey bees. *The XXXIst Int Cong of Apiculture*, Warsaw, Poland, 1987, Apimondia Publ, Bucharest, 217-220

Gilliam M, Valentine DK (1976) Bacteria isolated from the intestinal contents of foraging worker honey bees, *Apis mellifera*: the genus *Bacillus*. *J Invertebr Pathol* 28, 275-276

Gilliam M, Morton HL (1978) Bacteria belonging to the genus *Bacillus* isolated from honey bees, *Apis mellifera*, fed 2,4-D and antibiotics. *Apidologie* 9, 213-222

Gilliam M, Buchmann SL, Lorenz BJ (1984) Microbial flora of the larval provisions of the solitary bees, *Centris pallida* and *Anthophora sp. Apidologie* 15, 1-10

Gilliam M, Buchmann SL, Lorenz BJ, Roubik DW (1985) Microbiology of larval provisions of the stingless bee, *Trigona hypogea*, an obligate necrophage. *Biotropica* 17, 28-31

Gilliam M, Roubik DW, Lorenz BJ (1990) Microorganisms associated with pollen, honey, and brood provisions in the nest of a stingless bee, *Melipona fasciata. Apidologie* 21, 89-97

Machado JO (1971) Simbiose entre as abelhas sociais brasileiras (*Meliponinae, Apidae*) e uma especie de bacteria. *Cienc Cult (Sao Paulo)* 23, 625-633

Roubik DW, Michener CD (1985) Nesting biology of *Crawfordapis* in Panamá (Hymenoptera, Colletidae). *J Kans Entomol Soc* 57, 622-671