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# MICROBIAL FLORA OF THE LARVAL PROVISIONS OF THE SOLITARY BEES, *CENTRIS PALLIDA* AND *ANTHOPHORA* SP.

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## SUMMARY

All microbes isolated from the larval provisions of two solitary bees, *Centris pallida* and *Anthophora* sp. (an undescribed species in the Linsleyi group), were spore-forming bacteria belonging to the genus *Bacillus*. *Bacillus circulans* and *B. coagulans* were the most frequent isolates from provisions of *C. pallida*, and *B. circulans* was the only microbe found in the provisions of *Anthophora* sp. The bacteria isolated produced a variety of enzymes including esterases, proteases, amylases, and glycosidases. These findings are discussed in relation to the presence of *Bacillus* spp. in the food of other bees that are social. Since *Bacillus* spp. are well known for their wide range of metabolic activities and their ability to secrete their chemical products, they may be involved in the metabolic conversion, fermentation, and/or preservation of the food of bees.

## INTRODUCTION

Recently we examined the larval provisions of the eusocial stingless bee, *Trigona hypogea*, for microbes which might play a role in the production, metabolic conversion, and/or preservation of stored food (GILLIAM *et al.*, 1984). Five species of spore-forming bacteria belonging to the genus *Bacillus* were the only microorganisms found. *Bacillus pumilus* was the most frequent isolate followed by *B. megaterium*, *B. subtilis*, *B. circulans*, and *B. licheniformis*. Of interest was the fact that the same species of *Bacillus* were isolated from pollen of almond, *Prunus dulcis*, collected and stored in comb cells by honey bees, *Apis mellifera*, in Arizona (GILLIAM, 1979). It appeared that foraging honey bees added many of these bac-

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teria to the pollen as they moistened the grains to make a suitable mass to carry back to the hive since only *B. subtilis* was isolated from fresh pollen collected by hand from the flowers. Since *T. hypogea* uses vertebrate carrion as a protein source in lieu of pollen and since glandular provisions appear to replace stored pollen (ROUBIK, 1982), this striking similarity in the microbial complement of different types of food from two social bees may reflect either the ability of *Bacillus* spp. to survive in the particular microenvironments or similar metabolic roles of these microbes that have evolved in the nutrition of bees.

To test further these hypotheses, we report here the results of examination of the larval provisions of two additional species of bees, *Centris pallida* and *Anthophora* sp. (an undescribed species in the Linsleyi group). In contrast to *Apis* and *T. hypogea*, these bees are solitary and nest in soil. Both belong to the family Anthophoridae or « digger bees ». They are in the same sub-family but different tribes and are thus phylogenetically related. On the other hand, *Apis* and *Trigona*, both in the family Apidae, are closely related. All of these bees, except *Apis* use mass provisioning of cells ; *Apis* uses progressive provisioning.

Each female of *C. pallida* constructs a shallow nest which usually contains only one cell (ALCOCK *et al.*, 1976), or rarely two, and provisions the cell with many loads of pollen. These female bees are quite oligolectic in their choice of pollen for provisioning larval cells and use three species of desert legumes (two species of palo verde and ironwood) primarily. Both males and females are more catholic in the collection of floral nectar which is often gathered from plants other than palo verde or ironwood.

Once the larval cell is about one-third filled with pollen, the female adds a considerable amount of nectar. This is similar to the production of bee bread by honey bees. However, the larval provisions of *C. pallida* are liquid with the consistency of warm molasses. The female then oviposits floating the egg on the liquid surface, and the cell is then closed.

The *Anthophora* sp. that we examined had more than one large urn-shaped cell with a wax-like inner lining per nest. Cells of *Anthophora* characteristically have a thick wax lining. NORDEN *et al.* (1980) found that this lining in *Anthophora abrupta* is composed of liquid triglycerides produced by the Dufour's gland. These authors postulated that the triglycerides were enzymatically converted into solid diglycerides and that they function both as an impervious cell lining and as larval food. Freshly opened cells of large bees of *Anthophora* species have a « cheesy » or fermented odor which may be due to the waxy lining (NORDEN *et al.*, 1980) or microbial activity (MICHENER, 1974). *Anthophora* spp. provision cells in a manner similar to *C. pallida* but collect a greater diversity of pollen types (ROBERTSON, 1928).

Recently, CANE *et al.* (1983) showed in laboratory tests that volatile acyclic terpenoid and fatty acid derivatives released from the mandibular glands of female solitary bees during digging inhibited some fungi and bacteria associated with spoilage of provisions and diseases of larvae. Thus, the combination of antimicrobial substances of glandular origin and the lining of the cells protects the provisions from potentially harmful contaminants. Females of *C. pallida* have large Dufour's glands (ALCOCK and BUCHMANN, 1984), and they produce a cell lining.

## MATERIALS AND METHODS

Cells of *C. pallida* were collected 4 km north of Mesa, Arizona in May 1980. They were individually removed intact from the sandy soil and placed upright in sand in paper egg cartons for transport to Tucson in an air-conditioned automobile. Thus, the liquid provisions were prevented from mixing by running over the interior of the cells. Cells were placed in a freezer at the laboratory until they were analyzed.

We attempted to do as many analyses as possible on the larval provisions of the few available cells. Thus, 3 cells each were used to determine the following : weight of the individual cells including the provisions, percent total dissolved solids in the provisions by use of a hand refractometer, microscopic pollen identification, pH, volume of pure nectar in the uppermost layer, and moisture content of the provisions after drying samples at 60 °C for 3-4 days and then comparing weight differences. All cells used for analyses, including microbiological determinations, contained viable, apparently healthy eggs.

Cells of *Anthophora* sp. were removed from earthen nests located approximately 32 km east of Grand Junction, Colorado on the Colorado River in Garfield County in June 1980. They were hand-carried to Tucson via bus and thus were held at ambient temperature until they reached the laboratory where they were held in a freezer until analyzed. Robert W. BROOKS (University of Kansas, Lawrence) has determined that this bee is an undescribed species in the Linsleyi group and will describe it in a future publication.

Because of the small number of *Anthophora* sp. cells available, only moisture content of the provisions was determined. Cells used for all analyses contained apparently healthy eggs or first or second instar larvae feeding on the provisions.

Since microbiological examination of the provisions was only one aspect of the experiments involving *C. pallida* and *Anthophora* sp., we were able to plate provision material from one cell of each species. Cells were opened using aseptic conditions by removing the soil, swabbing the cell with 70 % ethanol, and puncturing the top of the cell with a sterile dissecting needle. A sterile capillary pipet was used to mix the provision material in each cell to avoid sampling only the nectar on the top or the pollen on the bottom.

Larval provisions from each cell were inoculated onto duplicate plates of nutrient agar (Difco), Czapek solution agar (Difco), and YM-1 agar (WICKERHAM, 1951) to isolate bacteria, yeasts, and molds. To test for the presence of anaerobes and microaerophilic organisms, duplicate tubes of thioglycollate medium without indicator (Difco) were inoculated. One plate or tube of each medium was then incubated at 25 °C and the other at 37 °C under aerobic conditions. During a 2-week incubation period, plates and tubes were examined periodically for microbial growth, and any colonies that developed were re-streaked onto plates of nutrient agar to test for and prepare pure cultures for identification. These plates were incubated at 37 °C.

Cell suspensions from all microbial colonies were stained by the Gram method and found to be spore-forming bacterial rods belonging to the genus *Bacillus*. This was confirmed by microscopic examination of the larval provisions. In this manner, the size, shape, and location of the spores within the sporangia were noted, and the morphology of the vegetative cells was determined. The organisms were maintained on slants of nutrient agar and were tested and identified according to the methods of GORDON *et al.* (1973) except that motility was determined in motility test medium (BBL) rather than microscopically.

Additional information on the enzymatic activity of each of the isolates was obtained by testing with the API ZYM system (Analytab Products) for 19 enzymes according to the manufacturer's directions.

## RESULTS

Provisions of *C. pallida* were bright orange due to the abundant palo verde pollen and filled one-third to one-half of the cell. The only pollen present belonged to both species of palo verde exploited by *C. pallida*. The provisions had a layer of moist but firm pollen at the bottom of the cell, a middle portion that was more liquid toward the surface, and a top layer of 100-200 µl of clear nectar with some floating droplets of lipids from the pollen below. The weight of the cells including the provisions ranged from 2.9-4.15 g, and the provision material amounted to 1-2 ml of liquid in each cell. The total dissolved solids ranged from 60-70 %, and the pH of the provisions was 5-6. The moisture content ranged from 15.3-16.3 % water.

Cell provisions of *Anthophora* sp. had a moisture content that ranged from 10.0-36.8 % with an average of 23.4 %. They were light orange in color and were homogeneous throughout with a thin layer of nectar of approximately 25 µl on the surface. These provisions had a fairly strong « cheesy » odor resembling that of butyric or isobutyric acid. The provisions of *C. pallida* had a mild « cheesy » odor that was barely detectable.

TABL. 1. — Bacillus *spp. isolated from larval provisions of* Centris pallida *and* Anthophora *sp.*

| Source | Organism | No. of isolates |
|---|---|---|
| *Centris pallida* | *Bacillus circulans* | 9 |
| | *B. coagulans* | 8 |
| | *B. firmus* | 1 |
| | *B. megaterium* | 1 |
| *Anthophora* sp. | *B. circulans* | 16 |

All microbes isolated from the larval provisions of *C. pallida* and *Anthophora* sp. were bacteria belonging to the genus *Bacillus* (Table 1). Thirty-five isolates

belonging to four species were identified. *Bacillus circulans* and *B. coagulans* were the most frequent isolates from provisions of *C. pallida*, and *B. circulans* was the only microbe found in the provisions of *Anthophora* sp. One isolate each of *B. firmus* and *B. megaterium* was also found in provisions of *C. pallida*.

Four atypical isolates of *B. circulans* were encountered. Three from provisions of *C. pallida* did not hydrolyze starch, and one from provisions of *Anthophora* sp. produced indole and did not hydrolyze starch. According to WILLEMSE-COLLINET *et al.* (1980), these reactions can occur with a small percent of *B. circulans* isolates, and GORDON *et al.* (1973) lists two strains of *B. circulans* as ± for starch hydrolysis. We prefer to recognize variability within a species rather than assigning new names to strains which differ in only one or two biochemical reactions from described species since in all other respects, our isolates of *B. circulans* conformed to the reactions given by GORDON *et al.* (1973).

All isolates of *Bacillus* spp. from provisions of *C. pallida* produced catalase and fermented D(+)-glucose with acid production only. Of the 19 isolates, 17 fermented D(+)-trehalose, 15 fermented (D—)-mannitol, and 14 fermented L(+)-arabinose and D(+)-xylose with acid production only. Seventeen produced acid in litmus milk, 16 hydrolyzed starch, 16 reduced nitrates to nitrites, 14 decomposed casein, 14 used citrate as the sole carbon source, 10 grew at pH 5.7, 12 grew in 5 % NaCl, 8 produced acetylmethylcarbinol, 8 produced dihydroxyacetone from glycerol, 3 grew in 7 % NaCl, and 3 liquefied gelatin.

Of the 16 isolates from provisions of *Anthophora* sp., all produced catalase and fermented D(+)-glucose and L(+)-arabinose with acid production only. Fifteen decomposed casein and hydrolyzed starch ; 14 fermented D(+)-xylose, D(—)-mannitol, and D(+)-trehalose with acid production only ; 11 decomposed tyrosine ; 10 reduced nitrates to nitrites ; 9 produced acid in litmus milk ; 7 used citrate as the sole carbon source ; and 4 grew in 5 % NaCl. None produced acetylmethylcarbinol, grew in 7 % NaCl, or grew at pH 5.7. Only one liquefied gelatin.

The enzymatic activities of the isolates as determined by the API ZYM system are shown in Table 2. One of the *B. circulans* isolates from provisions of *Anthophora* sp. died and was not tested. All isolates were negative for N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase, and all produced phosphoamidase.

Myristate lipase, cystine aminopeptidase, and β-glucuronidase were not produced by isolates from provisions of *C. pallida*. Most of the isolates from this source produced butyrate esterase, leucine aminopeptidase, caprylate esterase-lipase, and alkaline phosphatase. Most of the isolates from provisions of *Anthophora* sp. produced leucine aminopeptidase, chymotrypsin, and caprylate esterase-lipase.

TABL. 2. — *Results of tests for enzymes produced by* Bacillus *spp. isolated from larval provisions of* Centris pallida *and* Anthophora *sp.*

| Enzyme | No. positive/no. tested from | |
|---|---|---|
| | *C. pallida* | *Anthophora* sp. |
| Alkaline phosphatase | 16/19 | 5/15 |
| Butyrate esterase | 18/19 | 7/15 |
| Caprylate esterase-lipase | 17/19 | 12/15 |
| Myristate lipase | 0/19 | 2/15 |
| Leucine aminopeptidase | 18/19 | 14/15 |
| Valine aminopeptidase | 5/19 | 6/15 |
| Cystine aminopeptidase | 0/19 | 3/15 |
| Trypsin | 8/19 | 3/15 |
| Chymotrypsin | 6/19 | 14/15 |
| Acid phosphatase | 13/19 | 10/15 |
| Phosphoamidase | 19/19 | 15/15 |
| α-Galactosidase | 3/19 | 1/15 |
| β-Galactosidase | 4/19 | 7/15 |
| β-Glucuronidase | 0/19 | 1/15 |
| α-Glucosidase | 9/19 | 9/15 |
| β-Glucosidase | 2/19 | 3/15 |
| N-Acetyl-β-glucosaminidase | 0/19 | 0/15 |
| α-Mannosidase | 0/19 | 0/15 |
| α-Fucosidase | 0/19 | 0/15 |

## DISCUSSION

In the present study, the most frequent isolate was *B. circulans*. This bacterium has also been isolated from guts of worker honey bees, *A. mellifera* (GILLIAM and MORTON, 1978 ; GILLIAM and VALENTINE, 1976) ; from pollen from the corbiculae of foraging honey bees (GILLIAM, 1979) ; and from larval provisions of *T. hypogea* (GILLIAM *et al.*, 1984). *Bacillus coagulans* was a frequent isolate from provisions of *C. pallida* and has been found previously in the guts of both worker honey bees (EL-LEITHY and EL-SIBAEI, 1972 ; GILLIAM and MORTON, 1978 ; GILLIAM and VALENTINE, 1976) and mated queens (GILLIAM, 1978). *B. megaterium* has been isolated from guts of worker honey bees (EL-LEITHY and EL-SIBAEI, 1972 ; GILLIAM and MORTON, 1978), pollen from the corbiculae of foraging honey bees (GILLIAM, 1979), guts of virgin and mated queen honey bees

(GILLIAM, 1978), and now from larval provisions of *T. hypogea* (GILLIAM *et al.*, 1984). *Bacillus firmus* has been found in guts of worker honey bees (GILLIAM and MORTON, 1978 ; GILLIAM and VALENTINE, 1976).

Since *Bacillus* spp. were the only microorganisms found in the provisions of both *C. pallida* and *Anthophora* sp., they may play a role in the production, metabolic conversion, and/or preservation of the larval provisions of these bees. *Bacillus* spp. were also the only microbes found in the provisions of *T. hypogea*, bees which appear to digest dead animal tissue to obtain nutrient compounds that are then transported to the hypopharyngeal glands which produce the larval cell provisions (ROUBIK and BUCHMANN, unpublished).

MACHADO (1971) reported that a bacterium similar to *B. pumilus* appeared to predigest pollen collected by *Melipona quadrifasciata*. Elimination of the bacterium caused destruction of comb cells by the workers and the eventual death of the colony. The *Bacillus* appeared in large numbers only in the glandular secretion deposited on the pollen and honey layers in the cells. Furthermore, *Bacillus*, apparently of more than one species, was found in the larval food of 13 other species of bees in the sub-family Meliponinae (MACHADO, 1971).

Also, since *Bacillus* spp. were isolated from pollen and bee bread collected and stored by honey bees (GILLIAM, 1979), it seems possible that a special association between *Bacillus* spp. and bees has evolved. Female bees may inoculate food sources with microbes that are responsible for the metabolic conversion, fermentation, and preservation of food. The research cited certainly demonstrates the ability of these microbes to survive in the specialized environments and may indicate the resistance of *Bacillus* spp. to antimicrobial compounds such as those found in glandular secretions and nectar.

*Bacillus* spp. are well known for their wide range of metabolic activities and their ability to secrete chemical products. These abilities are exploited commercially. They produce many compounds including antibiotics (KATZ and DEMAIN, 1977), fatty acids (KANEDA, 1971) and numerous enzymes (BAPTIST *et al.*, 1978) such as pectinases, cellulases, amylases, proteases, β-glucanases, and isomerases. These bacteria secrete a number of extracellular enzymes in large quantities and usually produce several enzymes at the same time.

Results of the API ZYM tests revealed that the isolates from larval provisions of *C. pallida* and *Anthophora* sp. possessed a high activity of enzymes involved in lipid and protein metabolism and lower activity of glycosidases that hydrolyze carbohydrates. This was true also of the *Bacillus* spp. isolated from provisions of *T. hypogea* (GILLIAM *et al.*, 1984). Approximately half of the isolates in the present study produced α-glucosidase that hydrolyzes compounds such as sucrose, trehalose, melezitose, raffinose, and maltose. This is in contrast to the

production of this enzyme by only 2 of 15 isolates from provisions of *T. hypogea* (GILLIAM *et al.*, 1984) and probably reflects the higher concentration of these carbohydrates in provisions of *C. pallida* and *Anthophora* sp. However, isolates from *T. hypogea* had a higher activity of β-glucosidase that hydrolyzes carbohydrates such as cellibiose and salicin.

A higher proportion of the isolates from *C. pallida* compared to those from *Anthophora* sp. produced alkaline phosphatase, butyrate esterase, and trypsin. On the other hand, a greater percent of the isolates from *Anthophora* sp. produced chymotrypsin and β-galactosidase. Differing patterns of enzymatic activity by microbes from two sources may reflect adaptation to the chemical composition of specific food resources. Similar high activities were found for caprylate esterase-lipase, leucine aminoppetidase, acid phosphatase, and phosphoamidase by isolates from both sources as well as from *T. hypogea* (GILLIAM *et al.*, 1984).

Therefore, the results of both the API ZYM tests and the taxonomic tests showed that a wide variety of enzymes capable of acting on a variety of substrates was produced by the *Bacillus* spp. isolated. These enzymes include esterases, proteases, amylases, and glycosidases.

The production of both antibiotics and fatty acids by *Bacillus* spp. in larval provisions could limit the types of microbes that are able to survive, inhibit competition by other microbes for this food source, and protect the food from spoilage. The low pH of provisions may aid in this regard as well. Thus, these bacteria may be necessary for the maintenance of proper nutrition.

As previously stated, we are aware of the limitations of the small sample size used in this study but realized that we would be unable to obtain additional material from these species. In the future, we hope to examine provisions of additional species of bees to determine whether the association of *Bacillus* spp. with food sources is widespread among these insects.

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## RÉSUMÉ

### LA FLORE MICROBIENNE DES PROVISIONS LARVAIRES DES ABEILLES SOLITAIRES *CENTRIS PALLIDA* ET *ANTHOPHORA* SP.

On a recherché la présence de bactéries, de levures et de moisissures dans les provisions larvaires de deux abeilles solitaires, *Centris pallida* et *Anthophora* sp. (espèce non décrite du groupe Linsleyi), en les étalant sur un milieu microbiologique approprié, et aussi en examinant directement au microscope le matériau des provisions. Tous les microbes trouvés sont des bactéries qui forment des spores et appartiennent au genre *Bacillus*. *Bacillus circulans* et *B. coagulans* sont les espèces les plus fréquemment isolées des provisions de *C. pallida ; B. circulans* est le seul microbe trouvé dans les provisions d'*Anthophora* sp.

Les résultats de 19 tests enzymatiques utilisant le système API ZYM montrent que les espèces de *Bacillus* isolées possèdent une activité enzymatique élevée impliquée dans le métabolisme des lipides et des protéines et une activité plus faible des glycosidases, qui hydrolysent les hydrates de carbone. Une proportion plus grande de bacilles provenant de *C. pallida*, par rapport à ceux provenant d'*Anthophora* sp., produit de la phosphatase alcaline, de la butyrate esterase et de la trypsine. Pourtant un pourcentage plus élevé de bacilles venant d'*Anthophora* sp. produit de la chymotrypsine et de la β-galactosidase. Les bacilles des deux origines montrent une activité semblablement élevée pour la caprylate esterase-lipase, la leucine aminopeptidase, la phosphatase acide et la phospho-amidase.

Ces résultats sont discutés en rapport avec le large spectre d'activités métaboliques de *Bacillus* spp. d'une part et, d'autre part avec la présence de *Bacillus* spp. dans la nourriture d'autres abeilles, qui sont sociales. Il se peut qu'une association particulière se soit développée entre les abeilles et *Bacillus* spp. et ces microbes ont pu jouer un rôle dans la conversion métabolique, la fermentation et/ou la conservation des sources alimentaires.



## ZUSAMMENFASSUNG

### DIE MIKROBENFLORA DES LARVENFUTTERS DER SOLITÄREN BIENEN *CENTRIS PALLIDA* UND *ANTHOPHORA* SP.

Das Larvenfutter von zwei solitären Bienen, *Centris pallida* und *Anthophora* sp. (eine unbeschriebene Art aus der Linsleye Gruppe) wurde auf Bakterien, Hefen und Schimmelpilze untersucht, und zwar durch Ausstriche auf geeignete Nährböden und durch direkte mikroskopische Untersuchung des zur Fütterung eingelagerten Materials. Alle aufgefundenen Mikroben waren sporenbildende Bakterien aus dem Genus *Bacillus*. *Bacillus circulans* und *B. coagulans* waren die häufigsten Isolate aus den Futtervorräten von *C. pallida*. *B. circulans* war die einzige Mikrobe, die in dem Futter von *Anthophora* gefunden wurde.

Die Versuche mit API-ZYM-System Tests, die 19 Enzyme erfassen, ergaben bei den Isolaten von *Bacillus* spp. eine hohe Enzymaktivität im Lipid- und Protein-Metabolismus und eine geringere Aktivität der Glukoxidase, die Kohlehydrate hydrolysiert. Ein größerer Prozentsatz der Isolate von *C. pallida* als von *Anthophora* sp. produzierte basische Phosphatase, Butyrat-Esterase und Trypsin. Dagegen erzeugte ein größerer Anteil von Anthophora-Isolaten Chymotrypsin und β-Galactosidase. Bei Isolaten von beiden Arten wurden ähnlich hohe Aktivitäten für Caprylat-Esterase-Lipase, Leucin-Aminopeptidase, saure Phosphatase und Phospho-Amidase festgestellt.

Diese Ergebnisse wurden in Bezug auf das breite Wirkungsspektrum der Stoffwechselaktivität von *Bacillus* spp. und in Hinblick auf das Vorhandensein von *Bacillus* spp. im Futter von Bienen mit sozialer Lebensweise diskutiert. Es könnte sich eine spezifische Beziehung zwischen Bienen und *Bacillus*-Arten entwickelt haben, und diese Mikroben könnten in der metabolischen Umwandlung, der Fermentierung und/oder der Konservierung der Nahrung eine Rolle spielen.

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