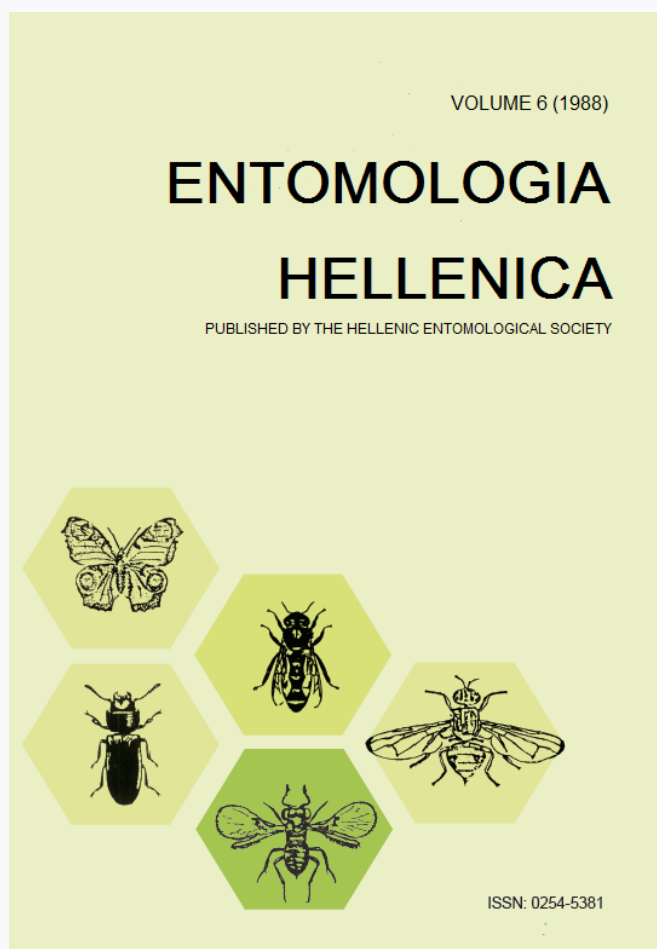


# ENTOMOLOGIA HELLENICA

Vol 6 (1988)



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doi: [10.12681/eh.13957](https://doi.org/10.12681/eh.13957)

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### To cite this article:

Stamopoulos, D. C., & Tzanetakis N. M. (1988). Bacterial flora isolated from the oesophageal bulb of the olive fruit fly *Dacus oleae* (Gmelin). *ENTOMOLOGIA HELLENICA*, 6, 43–48. <https://doi.org/10.12681/eh.13957>

# Bacterial Flora Isolated from the Oesophageal Bulb of the Olive Fruit Fly *Dacus oleae* (Gmelin)<sup>1</sup>

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## ABSTRACT

In a study of the bacterial flora occurring in the adults of the olive fruit fly, *Dacus oleae* (Gmelin) (Diptera: Tephritidae), oesophageal diverticulum, a total of 28 strains were obtained. Six of them were Gram - negative and identified as *Pseudomonas mendocina*, *Moraxella nonliquefasciens* (2), *Alcaligenes* sp., *Enterobacter cloacae* (2) and 22 Gram-positive classified as *Kurthia* sp., *Staphylococcus* subgroup VI, *Micrococcus roseus*, *Bacillus pumilus*, *B. licheniformis* (3) and *B. subtilis* (15). None of the above bacteria are strictly fixed and constantly present in the oesophageal diverticulum, suggesting that the bacterial flora associated with *D. oleae* depends on environmental factors, and could be used as a nutrient source for the insect apart from its possible other symbiotic role.

## Introduction

It has long been known that ectocellular bacteria are associated with many species of Dacinae and their presence is attributed to a symbiotic activity although direct evidence of a decisive contribution in the insect's metabolism is lacking (Tzanakakis 1984). In *Dacus oleae* (Gmelin) (Diptera: Tephritidae), these bacteria are housed in special structures of the alimentary tract of larvae and adults. In the latter, they are found mainly in an oesophageal diverticulum placed anterior to the brain and connected to the foregut. Petri (1910) was the first to report symbiosis of *D. oleae* with the bacterium *Pseudomonas savastanoi* Stevens a plant pathogen causing the olive knot disease. Hellmuth (1956) and Hagen (1966) confirmed Petri's findings but this association, however, was questioned by Yamvrias et al. (1970) who failed to isolate this bacterium from wild olive fruit flies. In 1974, Stamopoulos (unpubl.) tried

to verify indirectly the potential presence of *P. savastanoi* in the diverticulum of *D. oleae* inoculating by scarification small branches of olive trees with a culture of the fruit fly bacteria in Nutrient Broth (Difco); a culture of *P. savastanoi* obtained by incubation of olive tumours in the same medium was used as control. None of the branches inoculated with the insect's bacteria developed tumours while the control scarifications caused knots in all cases. Luthy et al. (1983), investigating oesophageal diverticulum by scanning electron microscopy, showed that this structure was filled with a pure population of an unidentified Gram - negative bacterium.

This paper reports preliminary experiments undertaken to elucidate the kind of bacteria occurred in the oesophageal bulb of *D. oleae* and to contribute in the investigation of the role they play.

## Materials and Methods

All head cysts investigated originated from adult insects obtained from field - collected pupae from the

<sup>1</sup> Received for publication January 31, 1989.

Halkidiki region (N. Greece). The emerged adults were kept in the laboratory in standard fruit-fly  $30 \times 30 \times 30$  cm cages and were fed a mixture of hydrolyzed yeast - sugar - water at a ratio of 1 - 4 - 5 by weight offered as droplets on wax paper; sugar and tap water were also available to the flies from the first day of their emergence. The temperature was  $25 \pm 2^\circ\text{C}$ , RH  $55 \pm 5\%$  and the daily photoperiod of 16h (fluorescent + natural light from a north - east oriented window).

The head capsules of about 60 three - day - old adults of both sexes were externally disinfected by dipping into 70% alcohol for 20 sec then rinsed with sterile water (Thuillier et al. 1967) and dissected in sterile droplets of lactate Ringer's solution under the binocular. The diverticulum was transferred aseptically with a needle to a Yeast Glucose Lemco Broth (Harrigan and McCance 1976) and incubated at  $28^\circ\text{C}$  aerobically until turbidity of the medium was ob-

served. All cultures obtained were spread with a loop on sterile Yeast Glucose Agar (Harrigan and McCance 1976) plates and incubated at  $30^\circ\text{C}$  for 48h. By repeated transfers to new agar plates we finally isolated a total of 28 strains which were kept as stock cultures at  $4^\circ\text{C}$ . For identification purposes, each strain was transferred to Yeast Glucose Agar and/or to Yeast Glucose Broth and incubated for 48h at  $30^\circ\text{C}$ . Gram staining, cell morphology, catalase and oxidase production, motility, flagellation, growth anaerobically, acid from glucose, Hugh and Leifson test, acid and gas from lactose and spores production were performed according to directions given either in the Manual of identification of medical bacteria (Cowan 1974) or in Bergey's Manual (Krieg and Holt 1984, Sneath et al. 1986). As classification tools we used the afore - mentioned manuals in addition to Identification Methods for Microbiologists (Gibbs and Skinner 1966, Skinner and Lovelock 1979).

TABLE 1. Characteristics of six strains of Gram - negative bacteria isolate from the oesophageal diverticulum of *D. oleae*. R = rods, Po = polar, Pe = peritrichus, F = fermentative, NT = not tested.

Characteristics	1 strain	2 strains	1 strain	2 strains
Shape	R	R	R	R
Catalase	+	+	+	+
Oxidase	+	+	+	-
Motility	+	-	+	+
Flagella	Po, 1	-	Pe	Pe
O/F test	-	-	-	F
Acid from glucose	-	-	-	+
Acid & Gas in McConkey broth	-	-	-	+
Indole production	-	-	-	-
Citrate	+	+	+	+
Nitrate	+	+	+	+
Growth at $4^\circ\text{C}$	-	-	-	-
Growth at $41^\circ\text{C}$	+	NT	-	+
Methyl red	-	-	-	-
Voges Proskauer	-	-	-	+
Urease	+	+	NT	1+, 1-
Esculine	NT	NT	NT	+
DNase	-	-	NT	-
Lipolysis	+	+	+	-
Casein hydrolysis	-	-	-	-
Gelatin hydrolysis	-	-	-	+
Lysine decarboxylase	-	-	-	-
Arginine dehydrolase	+	+	+	+
Starch hydrolysis	-	NT	NT	NT
Non-difusible pigments	-	NT	NT	NT
Growth on Nutrient Broth	+	+	+	+
Phenylalanine deaminase	NT	-	NT	-
Acid from:				
Lactose	NT	-	NT	*
Maltose	NT	-	NT	*
Xylose	NT	-	NT	*
H <sub>2</sub> S on TSI	NT	NT	NT	-
Ornithine decarboxylase	NT	NT	NT	+

*Ps. mendocina*

*M. nonliquefasciens*

*Alcaligenes* sp.

*E. cloacae*

\* Both strains ferment Lactose, Sucrose, Mannitol, L-arabinose, Raffinose, Ramnose, Maltose, Xylose, Trehalose, Cellobiose and Melibiose.

## Results and Discussion

From the 28 isolated strains, 6 were Gram negative (Table 1) and 22 Gram + positive (Table 2). From the Gram negative, 1 strain was characterized as *Pseudomonas mendocina* (Hendrie and Shewan 1979), 2 as *Moraxella nonliquefasciens* (Cowan 1974), 1 as *Alcaligenes* sp. (Cowan 1974) and 2 as *Enterobacter cloacae* (Richard 1984). From the Gram positive strains (Tables 2 and 3), 1 was characterized as *Kurthia* sp., 1 as *Staphylococcus subgroup VI* (Baird - Parker 1966), 1 as *Micrococcus roseus* (Baird - Parker 1966, 1979), 1 as *Bacillus pumilus*, 3 as *B. licheniformis* and 15 as *B. subtilis* (Claus and Berkeley 1986). After the above results, it seems that the microbial flora occurring in the head capsule of *D. oleae* adults, and especially in the oesophageal diverticulum, is not strictly fixed and seems to depend on factors not yet determined. Yamvriasis et al. (1970) arrived at similar conclusions as also Tsiropoulos (1976) who studied the bacterial flora of an other Trypetidae, the walnut husk fly *Rhagoletis completa*.

The presence of different bacteria and the fact that many of them are commonly found in the soil or in the dust can lead us to the

hypothesis that their presence in the insect oesophagus may be due to an accidental introduction during the feeding process, or, by accepting the theory established by Courtice and Drew (1984) and Drew and Lloyd (1987) for the Dacinae occurring in Australia, these bacteria constitute a natural food source for the adults. In fact, the adults of *D. oleae* have been observed many times "sponging" the glass or the bottom surface of their cages showing a regurgitation / reingestion behaviour similar to that of the Australian Dacinae fed on the bacterial flora occurring on the leaf surfaces of their host - plants (Courtice and Drew 1984). Although the feeding habits of *D. oleae* are different from those of the afore - mentioned fruit flies, it is not quite improbable to have similar necessities in bacteria that are served as food rather than as "obligate symbiotes". Toward this hypothesis is Girolami's observation (1973) that "the oesophageal bacteria of *D. oleae* escape periodically from the inside to the outside of the cephalic organ to fill most of the midgut, in small compact masses"; this function reminds a feeding rather than a symbiotic one. From recent investigations, it is also known that the enzymatic activities of the alimentary tract of *D. oleae* are almost the same in

TABLE 2. Characteristics of 22 strains of Gram-positive bacteria isolated from the oesophageal diverticulum of *D. oleae*. R = rods, F = fermentative, d = variable, C(1) = cocci in clusters, C(2) = cocci in pairs, tetrads, clusters, NT = not tested.

Characteristics	1 strain	1 strain	1 strain	19 strains
Shape	R	C(1)	C(2)	R
Catalase	+	+	+	+
Oxidase	-	-	-	d
O/F test	-	F	-	F
Acid from Glucose	-	+	-	+
Spores	-	-	-	+
Anaerobic growth	-	NT	NT	d
Motility	+	-	-	+
Coagulase	NT	-	-	NT
Acetoin	NT	+	-	NT
Acid from:				
Arabinose	NT	-	-	NT
Lactose	NT	+	-	NT
Maltose	NT	+	+	NT
Mannitol	NT	+	-	NT
Phosphatase	NT	-	-	NT
Pigment	NT	NT	Pink	NT
Growth in 10% NaCl	NT	NT	-	NT
	<i>Kurthia</i> sp.	<i>Staphylococcus</i> subgroup VI	<i>Micrococcus</i> <i>roseus</i>	<i>Bacillus</i> sp.

TABLE 3. Characterisation of the 19 strains of the genus *Bacillus*.

Characteristics	3 strains	1 strain	15 strains
Spores round	—	—	—
Sporangium swollen	—	—	—
Catalase	+	+	+
Growth anaerobically	+	—	—
VP	+	+	+
Acid from:			
D-glucose	+	+	+
L-arabinose	+	+	+
D-xylose	+	+	8+, 7W*
D-manitol	+	+	+
Gas from glucose	+	—	—
Hydrolysis of:			
Casein	+	+	+
Starch	+	—	+
Citrate utilisation	+	+	+
Nitrate red. to nitrite	W	—	13+, 2W
Growth at pH 5.7	+	+	+
Growth in NaCl:			
2%	+	+	+
5%	+	+	+
7%	+	+	+
10%	—	—	—
Growth at:			
7°	1+, 2—	—	+
30°	+	+	+
40°	+	+	13+, 2W
50°	—	+	—
65°	—	—	—
	<i>B. licheniformis</i>	<i>B. pumilus</i>	<i>B. subtilis</i>

\* W = week reaction.

“aposymbiotic” as in “normal” individuals (Stamopoulos unpubl.), and this leads to question the long believed theory that bacteria housed in the digestive system provide to the insect the necessary enzymes for its normal growth. On the other hand, the nutritional interpretation of the role of the digestive bacteria cannot explain by itself the observed inability of *D. oleae* larvae to grow in green olives when their parents received streptomycin and thus deprived them from the oesophageal bacteria (Fytizas and Tzanakakis 1966); so, it is possible that this bacterial flora could have a double role, serving as food and as a “producer” of substances useful to the insect.

It should not go unmentioned that, in spite of all the work done towards this subject, the problem of “*D. oleae* symbiote(s)” remains still perplex. Luthy et al. (1983) postulate that the oesophageal diverticulum harbours only one species of bacterium that is morphologically different (as it appears in scanning

microscope) from that described by Poinar et al. (1975) using the transmission electron microscopy. In our opinion, the image obtained by any kind of microscope is not by itself sufficient to answer such type of questions because many bacteria have similar external morphology. Furthermore, we cannot distinguish any existent difference when we examine the interior of the diverticulum, “in toto”. Finally, the same microorganism can present different morphological characteristics as Girolami (1973) observed in the case of *D. oleae* “symbiote”. On the other hand, if we try to answer why this observed diversification in bacteria species isolated from different workers exists we can only propose 2 possible explanations: first, the microbial flora associated depends on the insect’s environment, second, the real bacteria housed in the oesophageal diverticulum are difficult to cultivate and the isolated ones were only “occasional”, occurring on the external surface of the diverticulum and/or in



the foregut. The latter hypothesis seems to be less obvious because from previous work carried out by other investigators, it has been shown that the bacteria occurring in the Trypetidae and especially those of *D. oleae*, are easy to isolate using ordinary culture media (Yamvrias et al. 1970, Tsiropoulos 1976, Luthy et al. 1983, Rossiter et al. 1983, Drew and Lloyd 1987). The critical point would be, perhaps, the incubation temperature that according to Luthy et al. (1983) must not exceed the 28°C although our isolates seem to grow equally well at 28°C as at 30°C.

Nevertheless, in such type of work it is not improbable to isolate bacteria that are not housed systematically in the insects diverticulum. In fact, the vicinity of the cyst with different oesophageal fluids can lead to isolate microorganisms with no real symbiotic or other function for the insect and considered from this point of view as "contaminants". Only long term repeated isolations using insects of the same origin could give satisfactory responses in this dilemma under the presupposition of course that existence of bacteria strictly associated with the olive fruit fly really occurs. Further scrutiny of bacteria from different life stages and from insects of different geographical origin is now underway. The goal of this investigation is to determine the differences, if any, that exist among these isolated bacteria and to clarify their functional significance.

### Acknowledgment

We thank Professor M.E. Tzanakakis for his critical review of the manuscript.

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**KEY WORDS:** *Dacus oleae*, Olive fruit fly, *Dacus oleae* bacterial flora

## Μικροβιακή Χλωρίδα που Απομονώθηκε από την Οισοφαγική Κύστη του Δάκου της Ελιάς, *Dacus oleae*

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### ΠΕΡΙΛΗΨΗ

Από την οισοφαγική κύστη τελείων του *Dacus oleae* απομονώθηκαν 28 στελέχη διαφόρων βακτηρίων. Από αυτά, έξι ήσαν Gram-αρνητικά και ταυτοποιήθηκαν ως *Pseudomonas mendocina*, *Moraxella nonliquefasciens* (2), *Alcaligenes* sp., *Enterobacter cloacae* (2) και 22 Gram-θετικά τα οποία ταυτοποιήθηκαν ως *Kurthia* sp., *Staphylococcus* subgroup VI, *Micrococcus roseus*, *Bacillus pumilus*, *B. licheniformis* (3) και *B. subtilis* (15). Τα αποτελέσματα έδειξαν ότι κανένα από τα παραπάνω βακτήρια δεν απαντάται σταθερά στην οισοφαγική κύστη, πράγμα που μας οδηγεί στην υπόθεση ότι η μικροβιακή χλωρίδα του Δάκου εξαρτάται από διάφορους περιβαλλοντικούς παράγοντες. Εκφράζεται επίσης η άποψη, ότι η παρουσία των μικροοργανισμών αυτών πέρα από κάποιο συμβιοτικό ρόλο που μπορεί να παίζει, είναι δυνατόν να χρησιμεύει και ως πρόσθετη πηγή τροφής για το έντομο.