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Microbiology of the Oil Fly, *Helaeomyia petrolei*

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Helaeomyia petrolei **larvae isolated from the asphalt seeps of Rancho La Brea in Los Angeles, Calif., were examined for microbial gut contents. Standard counts on Luria-Bertani, MacConkey, and blood agar plates indicated ca. 2×10^5 heterotrophic bacteria per larva. The culturable bacteria represented 15 to 20% of the total population as determined by acridine orange staining. The gut itself contained large amounts of the oil, had no observable ceca, and maintained a slightly acidic pH of 6.3 to 6.5. Despite the ingestion of large amounts of potentially toxic asphalt by the larvae, their guts sustained the growth of 100 to 1,000 times more bacteria than did free oil. All of the bacteria isolated were nonsporeformers and gram negative. Fourteen isolates were chosen based on representative colony morphologies and were identified by using the Enterotube II and API 20E systems and fatty acid analysis. Of the 14 isolates, 9 were identified as *Providencia rettgeri* and 3 were likely *Acinetobacter* isolates. No evidence was found that the isolates grew on or derived nutrients from the asphalt itself or that they played an essential role in insect development. Regardless, any bacteria found in the oil fly larval gut are likely to exhibit pronounced solvent tolerance and may be a future source of industrially useful, solvent-tolerant enzymes.**

The number and variety of extreme environments occupied by microbes suggest that there are very few naturally sterile sites on earth. Because of this, we were intrigued by the biotechnology potential of any microbes found in the larval gut of the oil fly, *Helaeomyia petrolei* (formerly *Psilopa petrolei*). Thorpe (25) referred to the oil fly as "undoubtedly one of the chief biological curiosities of the world." The adults are found around natural oil seeps and in pools of viscous waste oil near commercial oil fields (10, 25). The larvae are exclusively found submerged in the oil, where they ingest large quantities of oil and asphalt. Even though the larval guts are visibly full of the petroleum, the larvae cannot subsist on oil alone. Rather, nutritional experiments (25) showed that the oil fly larvae quickly devoured any animal matter present in the oil. Under natural conditions, insects and other arthropods trapped in the sticky oil are the chief source of food (25). Furthermore, during the summer the surface temperature of the oil often rises to 37 to 38°C. Regardless of its presumed toxicity and temperature fluctuations, the larvae suffer no ill effects from the crude oil (25) as long as they can reach the surface of the oil to acquire air.

Petroleum technologists knew about oil pool maggots for many years prior to their being described by Coquillet (6) in 1899 as a new species of the Ephydridae genus *Psilopa*. The oil fly was later studied carefully by Thorpe (25, 26) but has been totally ignored by biologists since then. In particular, there have been no reports on the microbes associated with oil fly larvae. We decided to examine oil fly larvae obtained from the Rancho La Brea asphalt seeps in Los Angeles, Calif. This decision was made for two reasons. First, the oil flies are stably established in this location (10), having been observed continuously for more than a hundred years, whereas in other locations their appearance can be sporadic. A stable population of oil fly larvae in one location would be most likely to have arisen as a result of selected for a defined microbial gut flora. The presence of fossilized extinct animal remains at Rancho La Brea has led to estimations that these asphalt seeps have been in existence for at least 40,000 years (22). Second, the "oil" at Rancho La Brea is an extremely viscous asphalt, roughly one-third of whose composition by weight includes the high-molecular-weight branched molecules classified as asphaltenes. There is an abundance of microbes able to metabolize the straight-chain C_8-C_{16} hydrocarbons; however, there is still a shortage of microbes able to degrade either asphaltenes or polyaromatic hydrocarbons. The asphalt seeps at Rancho La Brea provide an ideal environment for the selection of such hydrocarbon-degrading organisms.

Furthermore, any hydrocarbon-degrading microbes isolated from oil fly larval guts would perforce be solvent tolerant since the guts are completely filled with asphalt. Environments containing high concentrations of organic solvents are considered extreme environments (1), and bacteria able to tolerate organic solvents have recently been recognized as a subgroup of the extremophiles (1). To the extent that solvent tolerance is beneficial for bioremediation, it would seem easier to start with solvent-tolerant microbes rather than to carry out time-consuming enrichments to get them (11). This expectation seemed reasonable since the oil fly larvae themselves tolerate 50% turpentine or 50% xylene (mixed with equal parts crude oil) with no apparent ill effects (25). Additionally, as originally discussed by Thorpe (25), the digestive enzymes of larvae or their microbial gut floras are active in environments heavily laced with aromatic petroleum compounds, and there has recently been a great deal of interest in enzymes which can maintain their activity in organic solvents (20). The present paper reports on the microbial content of surface-sterilized oil fly larvae obtained from the Rancho La Brea asphalt seeps and two other locations in southern California over the past 4 years.

(A preliminary account of this work appeared earlier [21]).

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MATERIALS AND METHODS

Collection and maintenance of oil fly larvae. Larvae were supplied by one of us (C.A.S.). Samples containing three to five *H. petrolei* larvae were shipped overnight in plastic camera film cases containing enough Rancho La Brea asphalt to cover the inner surface of the container. Shipping larvae in larger quantities of oil resulted in the death of the larvae. On arrival, the film containers were opened and placed on their side in large (150-mm-diameter) sterile plastic petri plates. Additional larva-free oil was used to fill the bottoms of the petri dishes to a sufficient depth for larval motility (1 to 2 cm). Less viscous oil supplements, such as linoleic acid, glycerol, and mineral oil, were not able to sustain larval viability; the larvae gradually showed decreased motility and died. The petri dishes containing the oil and larvae were enclosed in a 25- by 25-cm Styrofoam box that was sealed with plastic wrap to maintain the freshness of the volatile Rancho La Brea oil. Larvae were fed approximately 40 mg of egg meat medium (Difco, Detroit, Mich.) every other day. Larvae remained viable for at least 2 weeks under these conditions.

Surface sterilization. Larvae were removed from the oil with a sterile wooden stick. Active larvae cast a shimmering light on the surface of the oil, pinpointing their location for collection. Retrieval of inactive larvae was impossible. To ensure surface sterility, each of the larvae captured was placed in 1 ml of autoclaved linoleic acid (ca. 60%; Sigma, St. Louis, Mo.) and vortexed at low speed. The linoleic acid was replaced repeatedly and vortexing was continued until all oil was removed from the larvae. At this time, the larvae appeared translucent. Each larva was washed twice in filter-sterilized 70% ethanol for 4 min, immersed in a solution of 15% hypochlorite containing 0.1% (vol/vol) Tween 20 for 4 min, and then rinsed twice in sterile phosphate-buffered saline (PBS) containing 0.1% Triton X-100 or Tween 20 (pH 7.0) for 4 min. Most larvae remained active after this surface sterilization protocol, and these active larvae were used immediately. As a control for the effectiveness of surface sterilization, larvae were placed on Luria-Bertani (LB) agar plates and allowed to crawl for 1 to 2 min. If inactive, the larvae were rolled across the plate with sterile forceps. The plates were incubated at 37°C and examined after 24 h. No ($\leq$1) colonies were observed, except in those locations where the crawling larvae had excreted frass.

Bacterial counts. Larvae were placed in a sterilized handheld Potter-Elvehjem homogenizer containing 2.5 ml of sterile PBS (pH 7.0) and homogenized for 5 min or until all the larval contents appeared evenly suspended. Samples were diluted 10-, 100-, and 1,000-fold in PBS, and 0.1 ml was plated in triplicate. For the larvae collected in 1994 and 1995, five larvae were homogenized together and samples were plated onto a rich medium containing 0.02% yeast extract, 0.2% peptone, 0.02% NH_4NO_3, 0.01% glucose, 0.2% meat extract, and 1.2% agar (YEPM). For the 1997 experiments, single larvae were homogenized and plated onto LB agar (Miller) from Difco (lot 97500JK), 0.1× LB agar (Miller), MacConkey agar plus lactose with bromcresol purple from BBL (lot 907658), or blood agar from Remel, Inc., Lenexa, Kans. All plates contained 1.5% agar and were incubated at 37°C for 24 h. Total bacterial counts were conducted by direct epifluorescence microscopy with acridine orange as described by Murray et al. (18).

Rancho La Brea asphalt viable counts. A glob of asphalt was added to a preweighed sterile microcentrifuge tube so that its weight could be determined by difference. Sterile linoleic acid (1 ml) was added, and the tubes were heated to 37°C and vortexed extensively. Samples were diluted in PBS containing 0.1% Tween 20 and plated in triplicate on both nutrient agar and Trypticase soy agar plates.

Nitrogen-fixing organisms. The medium used to isolate any nitrogen-fixing organisms present was a modification of nitrogen-fixing marine medium (3). It contained 0.02 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2 \cdot 2H_2O$, 0.015 g of K_2HPO_4, 0.01 g of Na_2CO_3, 1.5 mg of citric acid, 1.5 mg of $FeCl_3 \cdot 6H_2O$, 0.25 mg of disodium potassium EDTA, 0.5 g of glucose, 0.5 ml of glycerol, and 5 g of Noble agar per 500 ml, in addition to 5 ml of a trace metals solution containing 1.43 g of H_3BO_3, 0.91 g of $MnCl_2 \cdot 4H_2O$, 0.11 g of $ZnSO_4 \cdot 7H_2O$, 0.04 g of $CuSO_4 \cdot 5H_2O$, 0.0015 g of $Na_2MoO_4 \cdot 2H_2O$, and 0.025 g of $CoCl_2 \cdot 6H_2O$ per liter. One whole larva was surface sterilized and homogenized as described above. Dilutions of 100- and 1,000-fold were made, and 0.1 ml of each was plated in triplicate on the nitrogen-free medium. Plates were incubated at 30°C for 48 h.

Assay for hydrocarbon oxidizers. Isolation of hydrocarbon oxidizers present in the larvae was done on the nitrogen-fixing medium outlined above with the addition of 0.1% hydrocarbon. The hydrocarbons assayed were palmitic acid, benzoic acid (sodium salt), linoleic acid, and naphthalene. With the exception of naphthalene, all hydrocarbons were incorporated directly into the agar of the nitrogen-fixing medium. For the naphthalene assay, nitrogen-fixing medium was poured into sterile glass petri plates. After inoculation, one naphthalene pellet was placed on the petri plate glass cover, and the plate was inverted and incubated. Larvae were sterilized and homogenized as described above. Ten- and 100-fold dilutions were plated in triplicate for each hydrocarbon and incubated at 30°C. For all plate counts, CFU were enumerated and, when applicable, cell morphology is described. Fourteen different colony morphologies were observed. Cultures were maintained on stock plates of tryptic soy agar and blood agar plates that were streaked for isolation every 3 weeks. Liquid cultures of each isolate were grown overnight in tryptic soy broth, after which 1 ml of culture was placed in cryovials containing 70 μl of dimethylsulfoxide and stored at −80°C.

TABLE 1. Measurement of oil fly larval gut pH[a]

Indicator dye	pK_a	Color of:		
		Midgut anterior	Midgut posterior	Hindgut
Bromophenol blue	3.85	Purple	Purple	Blue
Chlorophenol red	6.0	Red	Yellow-red	Red
Bromothymol blue	7.1	Yellow	Yellow	Yellow

[a] Larvae were collected from Rancho La Brea in 1994.

Growth characteristics of *H. petrolei* microbial isolates. A single isolated colony of each of the 14 representative isolates was chosen and aseptically transferred with a sterile toothpick onto blood agar, MacConkey agar plus lactose, tryptic soy agar, and LB agar in duplicate. Each plate was divided into 15 equal sections, one for each isolate and one to serve as the negative control. The same sterile toothpick was used to transfer a portion of a single colony onto all four media in the following order: blood agar, MacConkey agar plus lactose, tryptic soy agar, and LB agar. The toothpick was placed into the parent colony each time prior to depositing cells onto the plate. A single sterile toothpick was stabbed into each of the media at section 15. The plates were incubated at 30 and 37°C for 24 h.

Biochemical tests. Isolates were Gram stained, and a presumptive identification was obtained for each with the Enterotube II system from BBL, Becton Dickinson, Inc., and API 20E strips from bioMérieux Vitek, Inc., Hazelwood, Mo.

Fatty acid analysis. Nineteen representative isolates were chosen based on morphology and BBL Enterotube identification, isolated on appropriate media, and sent to MIDI Labs (Newark, Del.) for identification based on fatty acid composition.

RESULTS

Morphological examination of oil fly larval guts. The mean weight of 19 larvae sampled was 3.3 mg, with a standard deviation of 1.1 mg. The larvae ranged from 0.5 to 1 mm in diameter and from 5 to 12 mm in length. The large variation observed is likely due to the larvae examined being in multiple instars, although attempts to identify successive instars were unsuccessful. Once the viscous black oil was removed, the larvae were translucent, allowing direct observation of the larval gut. An extensively looped digestive tract occupying ca. 50% of the total larval volume was plainly visible. No ceca were found. The absence of ceca is important because, if present, they could have contained a distinct population of microbes, as has been observed for other insects (7). Dissection of the larvae exposed a small foregut that comprised only a minor percentage of the total gut followed by a highly looped midgut that represented the majority of the total gut. The hindgut was a short saclike section extending to the anus. The foregut appeared to function as an esophagus. Ingested petroleum entered the midgut as very dark boluses that dispersed and lightened as they passed through the digestive tract, appearing clear upon arrival at the anus.

Estimation of gut pH. Gut pH values for the translucent larvae were determined as described previously by Walther et al. (27) for *Aedes aegypti* larvae. The pH indicator dyes bromophenol blue, chlorophenol red, and bromothymol blue were fed to viable larvae. As the dyes passed through the anterior midgut, posterior midgut, and hindgut, the dye colors were observed by microscopy (Table 1). The color change of bromothymol blue from blue to yellow indicates a gut pH that does not exceed 6.5, while the red color with chlorophenol red (Table 1) indicates a gut pH of ca. 6.3 to 6.6. Thus, oil fly guts appear to have a slightly acidic pH. The larvae remained healthy throughout; they did not appear to be damaged in any way by the pH indicator dyes. Similar measurements could not be made for the gut redox potential because the redox indicator dyes tested were toxic to the oil fly larvae. Identifying a slightly acidic gut pH in healthy larvae is significant because

TABLE 2. Culturable bacteria associated with oil fly larvae

Sample site	Collection date (mo/yr)	*n*	No. of CFU[a]	Medium type
Rancho La Brea	4/94	15	$1.06 \times 10^5 \pm 0.36 \times 10^5$	YEPM
	6/94	15	$1.26 \times 10^5 \pm 0.26 \times 10^5$	YEPM
	10/94	15	$1.40 \times 10^5 \pm 0.5 \times 10^5$	YEPM
	7/97	3	$1.24 \times 10^6 \pm 9.7 \times 10^5$	Blood agar
		3	$4.26 \times 10^5 \pm 3.5 \times 10^5$	MacConkey agar + lactose
		3	$6.28 \times 10^5 \pm 4.5 \times 10^5$	LB agar
Santa Paula	4/95	20	$3.8 \times 10^5 \pm 1.7 \times 10^5$	YEPM
Ojai	4/95	10	$5.6 \times 10^5 \pm 3 \times 10^5$	YEPM
Rancho La Brea (oil)	6/94	4	$5.5 \times 10^3 \pm 2 \times 10^3$	YEPM

[a] All values are per larva, except that for the oil sample, which is per milligram. Values are means ± standard deviations.

many lepidopteran and dipteran insects have strongly alkaline larval guts, ranging from pH 10 to 12 (8), and we wanted our bacterial plating conditions to reflect the larval gut conditions accurately.

Quantifying the presence of microbes in the oil fly gut. Surface-sterilized larvae collected at three different sites (Rancho La Brea, Santa Paula, and Ojai in Los Angeles County, Calif.) over a period of 4 years were examined for the presence of culturable aerobes and facultative anaerobes (Table 2). Each of the larvae had between 10^5 and 10^6 CFU. The numbers were compared by analysis of variance. For the samples plated on YEPM, LB agar, and MacConkey agar, the results ($P = 0.11$) indicated no significant difference in culturable microbes per larva, based on either the sample site or time of collection. Somewhat higher bacterial counts were detected with blood agar plates (Table 2). In the October 1994 samples, acridine orange direct-count analysis of 10 larvae indicated that there were ca. $8.6 \times 10^5 \pm 1.1 \times 10^5$ total microbes per whole surface-sterilized larva. The same batch of larvae contained ca. 1.4×10^5 CFU per larva (Table 2). Therefore, the number of culturable microbes represented 16% of the total microbial population. This value is comparatively high for an environmental sample. Atlas and Bartha (4) concluded that counts obtained by direct epifluorescence microscopy are typically 2 orders of magnitude higher than counts obtained by cultural techniques.

By comparison, the oil from which the larvae were taken contained 5.5×10^3 CFU/mg, which, assuming that a 3.3-mg larva contains 1 mg of oil, represents 3.9% of the culturable bacteria. However, there is a problem in determining the significance of bacterial numbers in the oil. Repeated cycling of oil through the larval gut results in substantially increased bacterial counts. This point is nicely illustrated by comparing the counts found in newly emerged asphalt with no insects (30 CFU/mg), older asphalt exposed to the air and containing many insects (600 CFU/mg), and the oil in which the larvae had been swimming just prior to surface sterilization (5,500 CFU/mg). The first two counts were from oil taken in situ from the Rancho La Brea asphalt seeps.

As an additional control to be sure that the bacteria found in the larval gut did not originate with the nonsterile food provided to the larvae, this food (Difco's egg meat medium) was assayed for bacterial contamination. However, only 46 CFU/mg was detected on LB agar, which is equivalent to less than 0.013% of the viable population per larva. Furthermore, the bacteria in the egg meat medium were gram positive exclusively; only *Sarcina lutea* and a *Bacillus* sp. were found.

Comparison of plating media and colony morphology. The viable counts reported in Table 2 indicate the number of bacterial heterotrophs present. In this regard, it did not seem to matter whether the bacteria were plated on YEPM, MacConkey agar plus lactose, tryptic soy agar, or LB agar; the CFU detected were very similar. However, slightly higher numbers were found on blood agar plates (Tables 2 and 3). Blood agar plates had been found to be superior to peptone-based agars for the isolation of all Proteeae, including *Providencia* (13). Of the three media used to enumerate the 1997 samples, blood agar did give the highest number of CFU, while MacConkey agar plus lactose gave the greatest variety of colony morphologies (Table 3). Eight different colony morphologies were recognized (Table 3). After 24 h at 37°C, the 2-mm pale tan colonies were the most abundant morphology on both LB and MacConkey agar whereas a very small pinpoint colony was the most abundant morphology on blood agar plates (Table 3). Based on their colony morphology, 14 of the isolated colonies were chosen for identification. Of these 14, all but 1 (OF004B) formed colonies of equal sizes on each of the other nutrient-rich media used in the experiment whose results are shown in Table 3. OF004B did not grow on MacConkey agar. Thus, the CFU detected (Table 2) seem not to be influenced by the medium type chosen except for the small pinpoint translucent colony type (OF002L), which had a low plating efficiency on MacConkey agar and a high plating efficiency on blood agar (Table 3). Indeed, the differences in the total counts detected

TABLE 3. Frequency distribution of colony morphologies[a]

Colony type	No. and strain with colony type on:		
	LB agar	MacConkey agar	Blood agar
2-mm pale tan	4.55×10^5 (72%), OF001L	2.27×10^5 (53%)	4.81×10^5 (39%), OF006B
Pinpoint, translucent	1.44×10^5 (23%), OF002L	1.5×10^4 (3.5%	5.86×10^5 (47%)
2- to 4-mm pink-orange	83 (0.01%), OF003L	None	152 (0.01%)
1.5-mm dark gray	None	1.38×10^5 (32%)	1.13×10^5 (9.1%), OF005B and OF008B
1.5- to 2-mm gray with large white center	None	5×10^4 (11.7%), OF013M	5.1×10^4 (4.1%), OF007B, OF010B, and OF011B
1.5- to 2-mm gray with small white center	None	None	2.7×10^3 (0.22%), OF004B and OF009B
1-mm clear	None	4.35×10^4 (10.2%), OF014M	None
1.25-mm doughnut	None	2.15×10^4 (5%), OF012M	None
Total	6.28×10^5	4.26×10^5	1.24×10^6

[a] All bacterial samples were from the July 1997 collection. Plates were incubated at 37°C for 24 h. Percentages indicate how frequently that colony morphology was observed on all the plates of a given type used to determine the total CFU/larva. These were based on a total of 2,265 colonies for LB agar, 1,085 colonies for MacConkey agar, and 1,182 colonies for blood agar. The strain designations indicate which colonies were picked for the 14 bacterial isolates identified in Table 4.

TABLE 4. Identification of selected bacteria from oil fly larvae (1997 isolates)

Strain[a]	Enterotube result	Profile	MIDI result	Similarity index	Match	API 20E result	Profile	Match
OF001L	*Providencia rettgeri*	20603	*Proteus vulgaris*	0.817	Good	*Providencia rettgeri*	0074201	Good
OF002L	*Acinetobacter lwoffii* or *Pseudomonas maltophilia*	00000	*Yersinia pseudotuberculosis*	0.468	Fair	Nonenteric		
OF003L	*Acinetobacter anitratus*	00040	*Shewanella putrefaciens*	0.759	Excellent	Nonenteric		
OF004B	*Acinetobacter lwoffii* or *Pseudomonas maltophilia*	00000	*Enterococcus pesudoavium*	0.036	Poor	Nonenteric		
OF005B	*Providencia rettgeri* or *Providencia stuartii*	20607	Not done			*Providencia rettgeri*	0274311	Excellent
OF006B	No ID[b] available	20202	*Providencia rettgeri*	0.682	Good	*Providencia rettgeri*	0034001	Poor
OF007B	No ID available	20203	*Providencia rettgeri*	0.807	Good	*Providencia rettgeri*	0274101	Excellent
OF008B	Enteric group 60 or *Yersinia kristensenei*	22002	*Morganella morganii*	0.799	Good	*Morganella morganii*	0154000	Excellent
OF009B	*Providencia rettgeri*	20603	*Proteus myxofaciens*[c]	0.843	Good	*Providencia rettgeri*	0274310	Excellent
OF010B	No ID available	20203	*Proteus penneri*[c]	0.783	Good	*Providencia rettgeri*	0274301	Excellent
OF011B	*Providencia rettgeri* or *Providencia stuartii*	20607	*Proteus penneri*[c]	0.876	Good	*Providencia rettgeri*	0274101	Excellent
OF012M	*Providencia rettgeri*	20606	*Proteus vulgaris*[c]	0.825	Good	*Providencia rettgeri*	0274301	Excellent
OF013M	*Tatumella ptyseos*	20004	*Edwardsiella tarda*	0.588	Good	*Klebsiella pneumoniae* or *Yersinia enterocolitica*[d]	0014321	Doubtful
OF014M	*Providencia rettgeri* or *Providencia stuartii*	20607	Not done			*Providencia rettgeri*	0274301	Excellent

[a] The designations L, B, and M mean that the strain was originally taken from plates containing LB agar, blood agar, and MacConkey agar + lactose, respectively.
[b] ID, identification.
[c] This strain had *P. rettgeri* as the next most likely identification, with a similarity index of >0.750.
[d] Possible identifications were narrowed to these two bacteria because OF013M is nonmotile.

with the three media (Table 3) were primarily due to differences in the plating efficiency for this colony type.

Bacterial identification. Three systems were used for the presumptive identification of the 14 1997 isolates (Table 4). These were the BBL Enterotube II system, the API 20E system, and fatty acid analysis as conducted by MIDI Labs. The Enterotube system revealed that six of the isolates were either *Providencia rettgeri* or *Providencia stuartii*, and three were likely *Acinetobacter* isolates (Table 4). Three of the isolates could not be identified with the Enterotube system. Similarly, the API 20E system revealed that nine of the isolates were *P. rettgeri*, one was *Morganella morganii*, one was either a *Klebsiella* or a *Yersinia* isolate, and the other three (the three which had been identified as *Acinetobacter* isolates with the Enterotube system) were nonenteric (Table 4). OF005B had the same API 20E profile as *P. rettgeri* HM-1, which had been isolated by Jackson et al. (12) from an insect-pathogenic nematode. Based on their Gram stain, colony morphology, and metabolic characteristics, 12 of the 14 isolates were chosen for further classification based on their fatty acid profiles. Fatty acid analysis identified five of the isolates as *Proteus* species, two as *P. rettgeri*, one as *M. morganii*, and one as *Shewanella putrefaciens* (Table 4). Overall, the MIDI classification resulted in different identifications than those deduced by the Enterotube biochemical tests. They were, however, very close to the API 20E identifications, especially when one considers that the next most likely identification for strains OF009B through OF012M was *P. rettgeri* (Table 4). Taxonomically, *Proteus*, *Providencia*, and *Morganella* are very closely related genera (12).

Additionally, 95 bacterial isolates from the 1994 larval samples were studied, 94.7% (90 of 95) of which stained gram negative. Seven of these bacteria, taken from the dominant colony morphologies, were identified according to their fatty acid profiles (Table 5). Of these, two of the isolates were identified as *Enterobacter* isolates, two as *Hafnia alvei*, and one as *Acinetobacter radioresistens*. Although none of the organisms identified from samples taken in 1994 matched those from samples taken in 1997, all of the bacteria identified stained gram negative, and most of them were enteric bacteria (Tables 4 and 5).

Screening for nitrogen fixation and aromatic-hydrocarbon catabolism. Each of the larvae sampled in 1997 was examined for the presence of bacteria capable of nitrogen fixation. They each contained an average of 7.0×10^3 nitrogen-fixing bacteria. Thus, ca. 1% of the culturable bacteria were nitrogen-fixing bacteria. We also looked for bacteria with specific degradative capabilities. However, in no case did we find bacteria able to grow on benzene, toluene, naphthalene, anthracene, phenanthrene, chrysene, benzopyrene, or camphor as the sole source of carbon and energy. However, ca. 40% of the 1994 isolates were able to grow on short-chain alkanes such as dodecane, and all of the 1997 isolates were able to grow on hexane. The tests for possible degradation of aromatic compounds were also conducted on the basal medium of Stanier et al. (24), and thus, it is unlikely that the absence of growth was due to an unfulfilled metal or vitamin requirement. Many times low levels of bacterial growth were observed on the first passage through a catabolic test medium, e.g., phenanthrene only, but no growth was found on subsequent passages through the same medium. We believe that the low levels of growth found initially resulted from larval components released during homog-

TABLE 5. Identification of selected bacteria from oil fly larvae by fatty acid profile analysis[a]

Strain	MIDI result	Similarity index	Match
234	*Acinetobacter radioresistens*	0.869	Excellent
300	*Pseudomonas putida*	0.273	Poor
DOM 7	*Hafnia alvei*	0.783	Good
G32	*Klebsiella pneumoniae*	0.462	Fair
G38	*Hafnia alvei*	0.680	Good
G51	*Enterobacter taylorae*	0.861	Good
Mea 100	*Enterobacter agglomerans*	0.855	Good

[a] Single colony isolates from larvae collected in 1994 were sent to MIDI Labs for fatty acid analysis.

enization and carried over through the dilution series prior to plating.

DISCUSSION

We have shown that oil fly larvae in nature contain ca. 2×10^5 heterotrophic bacteria. This value seems roughly constant for larvae collected at three locations over a period of 4 years. The bacteria detected are neutrophiles, facultative or obligate aerobes, and nonsporeformers, and almost all are gram negative. No fungi were found. It is difficult to distinguish whether the bacteria found in an insect gut system are symbionts (persistent) or transients. However, we know that the detected bacteria grow in oil fly larvae because, on a per-weight basis, the number of bacteria in the oil inside the larval gut has roughly 100 to 1,000 times more CFU than would be expected from the bacterial load of the free oil ingested. Of course, cause-and-effect comparisons are difficult because the bacteria found in free oil may have resided previously in one of the larvae.

P. rettgeri and *Acinetobacter* spp. are the dominant bacteria identified in the 1997 samples. Nine of the 14 isolates were identified as *P. rettgeri*, and the 3 nonenteric isolates (OF002L to OF004B) are likely *Acinetobacter* organisms. Given the fact that nine different colony morphologies turned out to be *P. rettgeri*, it is reassuring that *Providencia* strains are well known to produce many distinct colony morphologies on agar media (13) and that *P. rettgeri* itself has five biotypes and 84 O antigens (13). Therefore, we do not believe that the bacteria detected are symbionts or clonal. The Enterotube and API identifications found five and six different metabolic profiles, respectively, among the bacteria designated *P. rettgeri* (Table 4). *Providencia* is an important human pathogen noted for its extreme resistance to common disinfectants, antibiotics, and heavy metals (13). Indeed, there are very few *Providencia* isolates obtained from nonhuman sources (13), although *P. rettgeri* has been isolated from an insect-pathogenic nematode (12). However, the prominence of *Acinetobacter* spp. among the bacteria isolated from oil fly larvae (Tables 4 and 5) comes as no surprise. They are among the most prevalent hydrocarbon-utilizing bacteria, and they grow well at oil-water interfaces (9). They are of industrial interest because they produce an extracellular polysaccharide emulsifier called emulsan which has proven useful for the in situ biodegradation of oil pollutants (9).

So far we have no evidence that the bacteria detected contribute to insect physiology. Only wild-type larvae were available for these experiments, and thus, we could not study germfree larvae, which would have been possible if we could have started with oil fly eggs. Additionally, we have no direct evidence that the bacteria detected metabolize any part of the thick asphalt in which they reside; we have been unable to detect gut bacteria able to metabolize aromatic compounds. This observation is consistent with Thorpe's conclusion (25) that oil fly larvae cannot subsist on oil alone. It is, of course, still possible that a consortium of gut bacteria would be able to degrade these aromatic hydrocarbons even though individual bacteria cannot. It is also possible that some of the nonculturable bacteria have aromatic-hydrocarbon-degrading ability.

Our current view of the bacteria found in oil fly larval guts is as follows. (i) Oil fly larvae are carnivores whose chief source of food is other insects or animals trapped in the sticky oil. (ii) As this food is digested, the nitrogen-rich nutrients released make the larval gut (pH 6.5) suitable for the growth of any bacteria which happen to be there. Thus, the bacterial numbers are ca. 1,000 times greater than in free oil (a low-nitrogen environment), and the bacteria detected grow nicely on peptone-based media (Table 3). The comparatively low number of nitrogen-fixing bacteria detected (ca. 1%) is consistent with the majority of the bacteria sharing a carnivore's high-nitrogen diet. (iii) Bacteria growing in the larval gut must tolerate the selective pressure of the oil which is also present. Thus, the bacteria found are almost exclusively gram negative. Dormant *Bacillus* spores might be present in the free oil, but their vegetative cells would still not be able to grow in the larval gut. (iv) The identity of the gram-negative bacteria found is not constant; it is not clonal, and it does not constitute a population of symbionts for the insect. Chapman (5) concluded that insects with straight, simple digestive tracts, i.e., no ceca, tend to contain the fewest microbes and that these microbes are probably acquired with ingested food. The oil fly larval gut is an open system, and the diversity of bacteria detected (Tables 4 and 5) reflects the diversity of bacteria initially present on the insects or animals which became trapped in the sticky oil, thus becoming food for the oil fly larvae.

In this regard, it is quite reasonable to find *P. rettgeri* in the carnivorous oil fly larvae. Jackson et al. (12), while studying the transfer of *Photorhabdus* spp. by insect-pathogenic nematodes, noticed that the majority of *Heterorhabditis* spp. contained a second bacterial species, *P. rettgeri*, which was itself highly pathogenic to insects. Thus, the *P. rettgeri* found in the oil fly larvae may have actively contributed to their initial introduction to the oil by killing their previous insect host. In this view, the bacteria found free in the oil are really transient members of the oil flora more than they are transient members of the larval gut flora. This explanation parallels the hypothesis put forward by Kjelleberg et al. (14) for bacteria found growing slowly or not at all in the water column, i.e., that they might grow much more rapidly in the guts of ingesting invertebrates.

We note that Thorpe's (25) description of oil fly larvae states "that the hind gut contains enormous numbers of bacteria-like bodies. They are Gram positive, and approximately 1 μm in length." Thorpe's conclusion that they were gram positive appeared to have been based on in situ staining of sectioned hindguts with Murray's toluidine blue (25). Our study detected gram-negative, not gram-positive, bacteria. The reason for this apparent contradiction has not been determined. It is possible that we would not have detected the gram-positive bacteria seen by Thorpe because they were nonculturable under our plating conditions. Alternatively, Thorpe (25) may have observed a cross section through the microvilli of the insect's gut cells. Such sections often exhibit regular cell structures 1 to 2 μm in diameter with no nuclei visible (17).

Lipophilic hydrocarbons are harmful to bacteria because they accumulate in membrane lipid bilayers, thus affecting the structural and functional properties of those membranes (23). Our finding that almost all of the bacteria in oil fly larvae are gram negative agrees with the generalization of Aono and Inoue (1) that gram-negative bacteria show higher levels of organic-solvent tolerance. It also agrees with the conclusions of Kramer et al. (15, 16) that only gram-negative bacteria tolerate high levels (5 to 25%) of anionic detergents such as sodium dodecyl sulfate (SDS). Since the cytoplasmic membrane is the primary site of cellular damage by both organic solvents (23) and SDS (19), the outer membrane serves to reduce the periplasmic concentration of these harmful chemicals to an acceptable level. For SDS, Nickerson and Aspedon used [^{35}S]SDS to show that *Enterobacter cloacae* cells growing in 5% SDS had periplasmic SDS levels of ca. 0.15% (19). The situation with organic solvents is likely similar. Aono et al. (2) observed that for *Escherichia coli* JA300 growing in the presence of *n*-hexane or cyclohexane, the outer membrane appeared intact and un-

damaged, but the cytoplasmic membrane was damaged such that cytoplasmic ribosomes had leaked into the periplasm. Thus, we hypothesize that bacteria which are adapted to oil fly larval guts (Table 3) should be an excellent source of enzymes which tolerate organic solvents. Their periplasmic enzymes should be moderately solvent tolerant, while their extracellular enzymes should be highly solvent tolerant.

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