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BACTERIAL DENSITY AND SURVEY OF CULTIVABLE HETEROTROPHS IN THE SURFACE WATER OF A FRESHWATER MARSH HABITAT OF *ANOPHELES QUADRIMACULATUS* LARVAE (DIPTERA: CULICIDAE)

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ABSTRACT. We examined surface water samples collected in September and October 1994 from a freshwater marsh habitat containing larval *Anopheles quadrimaculatus* mosquitoes. Bacterial densities in direct microscopic counts ranged from 9.7×10^5 to 1.3×10^7 cells/ml. Densities of cultivable bacteria on trypticase soy agar medium ranged from 1.0 to 1.5×10^5 cells/ml. The majority of 888 isolates were gram-positive rods (41%) followed by gram-negative rods (28%). Analysis of the cellular fatty acid profiles of 824 isolates using gas chromatography and Microbial Identification Systems TSBA (Rev. 3.60) library software grouped the bacteria into *Bacillus* spp. (35%), other gram-positive bacteria (16%), pseudomonads (15%), other gram-negative bacteria including mainly Enterobacteriaceae and Vibrionaceae (21%), and profiles not recognized (13%). Among 33 genera within these groups, the most common were *Bacillus, Pseudomonas, Aeromonas,* and *Arthrobacter.*

KEY WORDS *Anopheles quadrimaculatus,* larvae, freshwater marsh, bacteria

INTRODUCTION

Bacteria are commonly ingested food items of larval mosquitoes (Walker et al. 1988b, Merritt et al. 1990) and may sustain larval growth (Laird 1988, Merritt et al. 1992a, Sota and Kato 1994, Orduz et al. 1995, Wotton et al. 1997). The surface microlayer of aquatic habitats of anopheline larvae contains abundant concentrations of nonaggregated bacteria, particles of plant detritus associated with bacterial aggregates, and amorphous organic material (Walker and Merritt 1993, Maki and Hermansson 1994). Larvae of *Anopheles quadrimaculatus* Say congregate in vegetated zones of their habitats (Hess and Hall 1943, Walker et al. 1988a) and feed at the air–water interface by gathering food particles from the surface microlayer (Clements 1992; Merritt et al. 1992b, 1996). Although the presence of bacteria in larval feeding zones has been documented (Thiery et al. 1993, Walker and Merritt 1993, Orduz et al. 1995), very little information exists on the structure of the bacterial community and the significance of its components to larval nutrition (Laird 1988, Merritt et al. 1992a). Apart from nutritional aspects, some bacteria produce proteinaceous toxins that kill larvae and applications of recombinant technology to express these larvicidal genes in native bacteria are dependent on knowledge of the bacterial community (Thanabalu et al. 1992, Porter et al. 1993, Thiery et al. 1993, Orduz et al. 1995). Here, we endeavored to characterize the bacterial community in a freshwater marsh habitat of larval *An. quadrimaculatus* in Michigan.

MATERIALS AND METHODS

On September 13 and October 5, 1994, surface water was sampled from a permanent freshwater marsh (*ca.* 1 ha) among emergent vegetation including cattails (*Typha*), reeds (*Phragmites*), bulrush (*Scirpus*), and sedges (*Carex*). On each date, 30 water samples were collected from sampling points delineated 1 m apart on 3 parallel 10-m transects. At each point, approximately 20 ml of water was collected by dipping a sterile vial into the water surface. At each point, larval *An. quadrimaculatus* were sampled with a standard 450-ml mosquito dipper and the surface water temperature and water depth were measured. Water samples were transported on wet ice to the laboratory for direct microscopic counts (DMCs) and characterization of the heterotrophic bacterial community cultivated on trypticase soy agar (TSA) medium.

To estimate bacterial densities in water samples using the direct count method, a 2.0-ml aliquot from each sample was preserved in formalin (4%, v/v) and stored at 4°C. Later, an aliquot from each preserved sample was processed by the method described by Walker and Merritt (1993). Briefly, a dilute aliquot of each preserved sample was stained with DNA-binding 4′,6-diamidino-2-phenylindole (DAPI, Sigma Chemical Co., St. Louis, MO), passed through a black Nuclepore® filter (0.22 μm, Costar, Cambridge, MA), and bacteria retained on the filter were enumerated using epiflourescence microscopy (Porter and Feig 1980) under 1,000× magnification. Bacteria were categorized as rods, cocci, and vibriolike cells (i.e., curved rods) as they were counted. Total counts were converted to cell densities using a standard formula involving sample volume and dilution, filter area, number of fields examined, field size, and total number of particles counted (Kepner and Pratt 1994).

No more than 4 h after sampling the marsh, a

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1.0-ml aliquot from each water sample was serially diluted to 1:5, 1:10, and 1:100 in autoclaved phosphate-buffered saline (PBS; 0.13 M NaCl, 9.0 mM Na_2HPO_4, 0.8 mM $NaH_2PO_4 \cdot H_2O$, 1.6 mM KH_2PO_4, pH 7.2). From each of the serial dilutions, a 0.1-ml aliquot was spread on TSA medium (Becton Dickinson Co., Cockeysville, MD), incubated at 25°C in humidified air, and examined at 24, 48, and 168 h to record the number of colony-forming units (CFUs) that emerged. Autoclaved PBS diluent was plated on TSA as a control. Cultivable cell densities were averaged from the CFUs adjusted for dilutions and inoculum volume. Bacterial densities calculated from DMC and CFU analyses were transformed with $\log_{10}$ prior to *t*-test for comparison of bacterial densities between sampling dates.

Sixteen colonies per water sample were selected for isolation from the spread plates used in CFU analysis. To satisfy a standardized growth parameter of the identification system (see below), we selected both common and unusual colony types that appeared within 24–48 h of incubation. Colonies were isolated on fresh TSA media and incubated as before. Isolated colony morphologies were described by color, form, elevation, edge, and diameter. Distinctive characteristics such as iridescence, swarming, or odors were also noted. Wet mounts of each bacterial isolate were prepared, Gram stained, and examined microscopically, and the cell morphology was described. Subcultures in trypticase soy broth were suspended in sterile glycerol (>15%, v/v) and frozen at −70°C for preservation (Stead 1990).

Identification of the heterotrophic bacterial isolates was based on cellular fatty acid profiles (Vestal and White 1989, Sasser 1990, Stead et al. 1992, Paisley 1996, Smith and Siegel 1996). Fatty acid methyl esters (FAMEs) from pure cultures were prepared according to the protocols of the Microbial Identification Systems (MIS; Microbial Identifications, Inc., Newark, DE), except for the culture medium, which was TSA instead of trypticase soy broth agar (TSBA). Briefly, approximately 40 mg of bacterial cells from a pure culture were lysed and cellular fatty acids cleaved from lipids during saponification using a strong base in aqueous methanol while held in a boiling (100°C) water bath for 30 min. After cooling, cellular fatty acids were methylated with acidic, aqueous methanol while held in an 80°C water bath for 10 min. Then, FAMEs were extracted from the aqueous phase into an organic phase that consisted of a 1:1 solution of high-grade hexane and methyl *t*-butyl ether. To remove free fatty acids and residual reagents, the FAME extracts were washed with a mild base solution. The extracts were then transferred to glass vials, sealed, and stored at −20°C until processed by gas chromatography (GC) using an HP5890 gas chromatograph equipped with a flame ionization detector, an HP3396 integrator, and an HP7673A sampler (Hewlett-Packard Co., Palo Alto, CA). The GC column was an Ultra 2 fused-silica capillary column (0.2 mm by 25 m, crosslinked 5% phenylmethyl silicone; Hewlett-Packard). The initial column temperature was 170°C increasing 5°C/min to 270°C, the column flow rate was 0.5 ml/min, the injection port temperature was 250°C, and the detector temperature was 300°C. The carrier gas was ultra-high-purity hydrogen.

The chromatographic FAME profiles from our bacterial isolates were compared to the MIS software-based, TSBA (Rev. 3.60) library of known bacterial profiles, which assigned a primary genus and species identification subject to interpretation using a similarity index value from 0 to 1.0 and, if present, the index values of secondary identifications. According to the MIS protocol, an accurate identification of an unknown occurs when the similarity index is 0.5 or greater for the primary match and no additional matches occur within 0.1 index units (Paisley 1996). If additional identifications are within 0.1 index units of the primary identification, the result must be considered as a multiple match. However, a genus may still be assigned if it is consistent between the multiple match identifications. Therefore, we summarized the results from multiple matches as specifically as possible, that is, genus, family, or gram reaction group. Indices less than 0.3 indicate a match of the unknown to the most closely related known profile, but that the unknown bacteria may not be in the MIS library. When the unknown profile did not fit any of the profiles in the library, then MIS assigned it a no match designation.

RESULTS AND DISCUSSION

The surface water temperature at the study site was 27°C on September 13 but decreased to 12°C on October 5. The water depths at the sampling points ranged from 20 to 30 cm on September 13 but increased to 28 to 40 cm on October 5 due to a total of 7.8 cm of intermittent rain that fell in the area between September 26 and October 2. The mean number of *An. quadrimaculatus* larvae per dip was 1.2 on September 13 and 0.07 on October 5.

Total bacterial cell densities determined by DMCs ranged from 1.0 to 13.0 × 10^6 cells/ml (Table 1), which was consistent with many other lentic systems (Fry and Zia 1982, Kirchman and Mitchell 1982, Danos et al. 1983, King et al. 1991). The density of cocci decreased significantly in the 2nd collection (*t*-test, $t = 3.5$, df = 57, $P < 0.05$) but no significant effect was found of collection date on the densities of total cells, rod-shaped bacteria, or vibriolike cells (*t*-tests, $P > 0.05$). The trend toward decreases in bacterial densities may have reflected temporal changes related to lower water temperature and dilution by rainfall. However, rainfall also generates soil runoff, which contributes influxes of nutrients and bacteria into aquatic systems

Table 1. Mean bacterial densities × 10^6 cells/ml ± standard deviation (range) estimated by direct microscopic counts and colony-forming units (CFUs) from surface water samples of a marsh habitat of *Anopheles quadrimaculatus* larvae in Michigan.

	Collection date	
	September 13	October 5
Rods[1]	3.3 ± 2.1 (0.6–7.4)	2.4 ± 0.8 (0.3–3.4)
Cocci[2]	2.8 ± 1.4 (0.6–5.4)	1.7 ± 1.2 (0.1–3.8)
Vibriolike cells[1]	0.4 ± 0.4 (0.0–1.6)	0.7 ± 0.6 (0.0–2.2)
Total cells[1]	6.5 ± 3.8 (1.2–13.0)	4.8 ± 2.0 (1.0–8.1)
CFUs[1]	0.1 ± 0.08 (0.03–0.4)	0.1 ± 0.07 (0.01–0.3)

[1] Densities between collections were not significantly different.
[2] Densities between collections were significantly different (t-test, $t = 3.5$, df = 57, $P < 0.05$).

(Reynolds 1989, Höfle 1992, Horne and Goldman 1994).

The densities of bacteria cultivable on TSA medium estimated by CFUs ranged from 1.0×10^4 to 4.0×10^5 cells/ml (Table 1). No significant differences were found in CFU densities between collection dates (t-tests, $P > 0.05$). In each sample, the proportion of cultivable cells estimated by CFUs ranged from 0.6 to 14.5% (mean 4%) of the total cell densities estimated by DMCs. Other types of cultivation media may have increased or decreased the density of bacteria recovered but compared to direct cell counts, cultivation techniques significantly underestimate total cell densities (Buck 1979, Kepner and Pratt 1994).

We selected a wide range of colony types for isolation to help describe the broad spectrum of cultivable bacteria present in the study site. Of 960 bacterial colonies selected for isolation, 888 (92%) could be maintained on TSA medium. Microscopic examinations found predominantly rods (73%), followed by coccobacilli (25%) and cocci (2%). Slightly more than one half of the isolates were gram-positive (53%), followed by gram-negative (41%) and gram-variable (6%) isolates. The majority of isolates were gram-positive rods (Fig. 1). No effect of collection date was found on the frequency of cultivable cell types or Gram stain groups (chi-square tests, $P > 0.05$). Rods accounted for 73% of cultivable cell types but only 50% of cell types in direct counts. Although cocci accounted for about 40% of the cell types in direct counts, discrete cocci were just 2% of cultivable isolates. The higher density of cocci seen in direct counts likely included dormant or starved bacteria that adapted to the stress of low nutrient conditions in the aquatic environment by decreasing cell volume to form microcysts or round bodies observed in several studies reviewed by Roszak and Colwell (1987). When cultured in higher nutrient conditions of TSA, cell volumes increased producing more diverse cell types, which likely included rod forms.

Of the 888 bacterial isolates that could be maintained on TSA, 830 (93%) yielded sufficient quantities of cellular material required by the MIS protocol to generate FAME profiles. Of 830 FAME profiles, 112 (13%) were not matched with any profiles in the MIS library. Because only a small portion of the bacteria in nature have been classified (Gottschal et al. 1992, Holt 1994), controversial identifications and unidentifiable isolates were expected from our study. Of the 718 matches, 641 were assigned a primary generic identification (at varying similarity indices) without a confounding

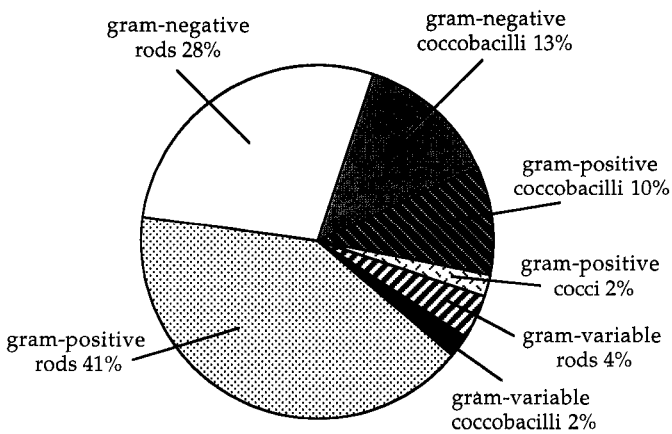

Fig 1. Gram reaction and microscopic morphologies of 888 bacterial isolates from a freshwater marsh in Michigan.

Table 2. Bacterial isolates from a marsh habitat of *Anopheles quadrimaculatus* larvae in Michigan assigned to gram-positive genera by the Microbial Identification System based upon analysis of cellular fatty acid profiles, and listed by similarity values and total number.

Genera	No. of isolates by similarity value			Total no.
	>0.5	0.5–0.3	<0.3	
All gram-positive genera	188	111	123	422
Bacillus	151	83	54	288
Arthrobacter	2	4	23	29
Brevibacterium		1	21	22
Cellulomonas	6	2	4	12
Staphylococcus	6	3	1	10
Clavibacter	5	3		8
Curtobacterium	6	2		8
Aureobacterium	4	2		6
Micrococcus	4		2	6
Corynebacterium		2		2
Nocardia			1	1
Rhodococcus			1	1
Multiple matches	4	9	16	29

Table 3. Bacterial isolates from a marsh habitat of *Anopheles quadrimaculatus* larvae in Michigan assigned to gram-negative genera by the Microbial Identification System based upon analysis of cellular fatty acid profiles, and listed by similarity values and total number.

Genera	No. of isolates by similarity value			Total no.
	>0.5	0.5–0.3	<0.3	
All gram-negative genera	83	74	139	296
Pseudomonadlike	31	26	70	127
Pseudomonas	29	22	60	111
Hydrogenophaga	1	2	4	7
Stenotrophomonas[1]			5	5
Comamonas	1	2		3
Methylobacterium			1	1
Enterobacteriaceae	38	10	5	53
Pantoea[2]	8	3		11
Erwinia	5	1		6
Hafnia	2			2
Providencia	2			2
Edwardsiella		1		1
Yersinia			1	1
Multiple matches	21	5	4	30
Vibrionaceae	8	20	32	60
Aeromonas	8	20	19	47
Alteromonas			12	12
Vibrio			1	1
Other gram-negative genera	6	18	32	56
Flavobacterium	1	5	7	13
Acinetobacter	1	6	1	8
Agrobacterium	3	2	2	7
Cytophaga			7	7
Actinobacillus			1	1
Pasteurella		1		1
Sphingobacterium			1	1
Multiple matches	1	4	13	18

[1] Genus name changed from *Xanthomonas* in trypticase soy agar broth (TSBA) (Rev. 3.60) library.

[2] Genus name changed from *Enterobacter* in TSBA (Rev. 3.60) library.

secondary generic match. The remaining 77 isolates were assigned multiple matches consisting of closely related genera. There were 422 gram-positive isolates distributed among 12 genera, the majority of which were *Bacillus* (68.2%), followed by *Arthrobacter* (6.9%), *Brevibacterium* (5.2%), *Cellulomonas* (2.8%), and *Staphylococcus* (2.4%); additionally, there were 29 (6.9%) gram-positive isolates matched to more than one genus (Table 2). There were 296 gram-negative isolates distributed among 21 genera; the majority of these were pseudomonadlike (42.9%), followed by Vibrionaceae (20.3%), Enterobacteriaceae (17.9%), and other gram-negative bacteria (18.9%) (Table 3). There were 30 isolates with multiple generic matches within Enterobacteriaceae and 18 isolates with multiple matches consisting of other gram-negative genera (Table 3). Overall, the 830 MIS designations were distributed among *Bacillus* spp. (35%), other gram-positive genera (16%), pseudomonadlike genera (15%), other gram-negative genera (21%), and profiles not recognized (13%) (Fig. 2).

The high proportion of gram-positive isolates in our survey may have reflected a community influenced by influxes of soil runoff from rainfall or by perturbations of bottom sediments. Gram-positive rods, particularly *Bacillus,* have been the most prevalent bacteria isolated from soil (Alexander 1977, Slepecky 1992), lake bottoms (Ramsay 1977), and water samples from freshwater habitats (Niewolak 1972, Cherry et al. 1974, Orduz et al. 1995). Other freshwater bacteriologic studies isolated gram-negative rods, principally pseudomonads, flavobacteria, and Enterobacteriaceae (Wolters and Schwartz 1956; Strzelczyk et al. 1971; Ramsay 1976, 1977; Hossell and Baker 1979; King et al. 1991; Chand et al. 1992; Höfle 1992; Lee et al. 1994). These groups were present among our isolations, along with a variety of other gram-negative isolates. Other cultivation media may have expanded or narrowed the diversity of bacteria recovered.

There is a remarkable paucity of information describing bacterial communities present in larval mosquito habitats. This study demonstrated a wide range of morphologic and taxonomic diversity within the bacterial community of a larval *Anopheles* habitat. We identified only a fraction of the total bacteria present; the bacterial food sources for *Anopheles* larvae are presumably even more diverse. This diversity likely influences digestibility (demonstrated in other invertebrates [King et al. 1991]) and the capacity to support larval growth (Orduz et al. 1995). Suppression of the bacterial community delayed larval development in laboratory studies (Walker et al. 1997, Wotton et al.

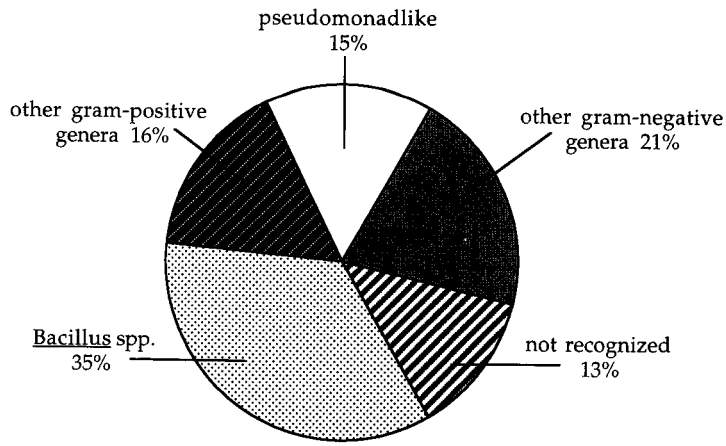

Fig 2. Categorizations of the Microbial Identification System (MIS) designations assigned to 830 bacterial isolates from a freshwater marsh in Michigan.

1997). The information presented here helps to provide a basis for future studies elucidating the contribution of specific bacterial taxa to larval mosquito diet.

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