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Bacterial Flora of the Schistosome Vector Snail *Biomphalaria glabrata*

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The aerobic heterotrophic bacterial flora in over 200 individuals from 10 wild populations and 3 laboratory colonies of the schistosome vector snail *Biomphalaria glabrata* was examined. Internal bacterial densities were inversely proportional to snail size and were higher in stressed and laboratory-reared snails. The numerically predominant bacterial genera in individual snails included *Pseudomonas, Acinetobacter, Aeromonas, Vibrio,* and several members of the *Enterobacteriaceae. Enterobacteriaceae* seldom predominated in laboratory colonies. Our data suggest that *Vibrio extorquens* and a *Pasteurella* sp. tend to predominate in high-bacterial-density snails. These snails may be compromised and may harbor opportunistic snail pathogens.

Human schistosomiasis (bilharzia) is a tropical parasite infection in which trematode flatworms of the genus *Schistosoma* infect blood vessels of the bladder or intestine. It is estimated that more than 200 million people are infected worldwide (6). The intermediate hosts of schistosomes are aquatic or amphibious snails from several widespread tropical genera. The principal host in the western henisphere is the freshwater planorbid pulmonate *Biomphalaria glabrata.* This snail is responsible for schistosomiasis transmission in Brazil, in Venezuela, and throughout the Caribbean region (22).

Transmission of schistosomiasis is usually controlled by the application of copper- or tin-based molluscicides (4). Biological control of snail populations offers inexpensive and environmentally acceptable alternatives to chemical molluscicides, but has been effective in only isolated instances (13). Microbiological control of snails has not been extensively studied (10). In fact, little is known about the microbiology of *Biomphalaria* spp. and other freshwater snails.

We are attempting to obtain bacterial predators of *B. glabrata* and evaluate them as biocontrol agents against schistosomiasis. Recently our efforts have centered on the role of opportunistic pathogens (21) in compromised hosts as a means of controlling snail populations. We are also studying the microbial ecology of wild natural and cultured laboratory populations of *B. glabrata* in order to elucidate some of the factors involved in the induction of snail diseases. We have found that experimental infection of *B. glabrata* with *Vibrio parahaemolyticus* can result in 100% mortality (H. W. Ducklow, H. Tarraza, and R. Mitchell, submitted for publication). We have also studied the quantitative relationships between snail size and bacterial population densities and have shown that opportunistic snail pathogens can be isolated from atypically colonized, compromised snails. The discovery of snails harboring opportunistic pathogens in wild populations suggests a new approach to the microbiological control of vector snails. In this report, we characterize the numerically predominant bacterial populations in individuals of *B. glabrata* from Puerto Rico, St. Lucia, and Guadeloupe and from our laboratory aquaria. Several of the predominant species are opportunistic pathogens which we have isolated for further studies on biocontrol tactics.

MATERIALS AND METHODS

Field studies and snail collection. We collected and bacteriologically analyzed individual snails from wild *B. glabrata* populations in Puerto Rico in June 1977 and in St. Lucia and Guadeloupe in July 1978. Snails were collected from a range of typical snail habitats and disease transmission loci on each island by using screen dippers (25 by 25 cm) (12). No attempts were made to sample a given site exhaustively. Rather, each site was sampled until enough snails were collected for analysis on the same day. *B. glabrata* individuals were removed from the screen dippers and transported to the laboratory in presterilized Whirl-Pak plastic bags (Nasco, Inc., Fort Atkinson, Wis.).

Sampling from aquarium snail colonies. Laboratory snail colonies were derived from the NIH-AID albino strain of Puerto Rican *B. glabrata* or from wild *B. glabrata* collected in St. Lucia and Guadeloupe. These snails were reared in 10- or 15-gallon (37.9- or

56.8-liter) glass aquaria equipped with undergravel filtration and fed washed romaine lettuce three times each week. To sample laboratory colonies, individuals from each aquarium were removed with alcohol-flamed forceps.

Bacteriological analysis. Each snail was examined for its colony-forming bacterial content by the following procedure. Our sampling procedures were optimized during initial analyses of aquarium populations of *B. glabrata* at Harvard. Only intact snails capable of locomotion were sampled. To estimate wet tissue weight of the snails, we measured the shell diameters of the first 16 snails collected in Puerto Rico and then weighed each snail after removing its shell. All subsequent snail fresh weights were based on the resulting regression of tissue weight on shell diameter.

All experimental procedures were carried out by aseptic technique. Each snail was placed in a sterile disposable petri dish, measured, and gently crushed between two autoclaved slides. The shell fragments were removed, and the snail body was transferred to a sterile glass Ten Broeck tissue grinder containing 9 ml of 0.1% proteose peptone in 0.0375 M phosphate buffer, pH 7.2. Each grinder also contained about 1 g of washed, precombusted (600°C) quartz sand to facilitate grinding. The snails were ground with an alcohol-flamed glass pestle until a homogeneous suspension was attained. The homogenate was mixed for 1 min at high speed on a Vortex mixer. Serial decimal dilutions were carried out from the homogenate into 9.0-ml dilution blanks containing the buffered peptone medium. Finally, 0.1-ml portions of the predetermined suitable dilutions were inoculated onto duplicate agar plates containing standard methods agar (TGY) containing in grams/liter: Trypticase peptone, 5.0; yeast extract, 2.5; dextrose, 1.0; and agar, 15.0 (BBL Microbiology Systems, Cockeysville, Md.). In our studies of laboratory snails, we found this medium to be preferable to Trypticase soy agar (BBL), nutrient agar, 0.1% peptone-yeast extract agar, and unamended agar (unpublished data). The plates were incubated at room temperature for at least 1 week before the colonies were counted.

After returning to Cambridge, we isolated the predominant colony types from each plate. No attempt at extensive random sampling was made. The plates were carefully examined under reflected and transmitted fluorescent light, and the characteristics of the numerically predominant colonies were noted. One or two typical colonies of the predominant type were isolated from each plate. Counts of the number of this colony type on each plate were made so that data on the populations of various isolates in the snails could be derived. To verify our colony selection criteria, all the colonies of each predominant colony type from several snails were isolated for further identification.

All isolates were purified by repeated streaking on TGY plates and maintained on TGY agar slants in screw-topped tubes. Of the isolates from each collection, 10 to 20% died after several transfers, so the collections are not representative of the overall colony-forming populations in *B. glabrata*. The surviving isolates were characterized as to Gram reaction and morphology, and the gram-negative rods were identified to the species or genus level by using Oxiferm tubes (Roche Diagnostics, Nutley, N. J.) or the API 20E diagnostic system (Analytab Products, Inc., Plainview, N. Y.). Isolates were assigned to the Oxiferm or API system on the basis of results from dextrose oxidation-fermentation and triple sugar iron agar tests carried out in conventional tubed media (Scott Laboratories, Fiskeville, R. I.). All identifications from the API and Oxiferm identification manuals were checked against criteria listed elsewhere (3, 15, 23). In several instances, other conventional tubed media were used in place of the rapid identification systems.

Comparison of aquarium snail and water populations. To assess the influence of the bacterial populations in the water on bacterial densities in snails, bacterial analyses of snails and water were carried out weekly over a 7-week period. Each week, two snails and two water samples were analyzed from each of two tanks. In one tank, the snail population consisted of snails ranging in size from about 2 to 7.0 mm in diameter. Snails larger than 7.0 mm were removed weekly. In the other aquarium, snails ranged in size from 7.0 to over 18 mm in diameter. Thus two snail samples, one of large and one of small snails, were made. The bacterial analyses were performed exactly as described above.

Transfer experiments. Two experiments testing the effects of environmental changes on wild or laboratory snails were carried out. Snails collected at Borricaud, Grande Terre, Guadeloupe, were returned to Cambridge and used to initiate a new snail colony. After 3 months, the original 20 snails had produced several hundred offspring. Ten first-generation offspring were sampled to assess the effect of aquarium cultivation on the microflora of genetically similar populations of host snails.

In a similar experiment, snails from Soufriere, St. Lucia, were suspended in a Nitex screen (Tetko, Inc., Elmsford, N.Y.) cage for 24 h at Morne Panache, St. Lucia, and then returned to Cambridge 24 h after removal from the test site. These snails were sampled as described above within 2 to 3 days after their arrival in Cambridge.

Axenic culture of laboratory snails. As part of another study, we cultured axenic albino *B. glabrata* by the procedures described elsewhere in detail (5).

Successfully maintained axenic snails were to be used in studies of monoassociation with various bacterial species and in the screening of snail pathogens. Briefly, the process involves sterilizing individual snail eggs in Triton N-100 and NaOCl, followed by culture in vitro in the presence of penicillin. Several of our axenically reared snails eventually were contaminated during feeding or transfer to fresh medium. After 2 months of cultivation in the contaminated state, these snails were sacrificed and sampled as described.

RESULTS

Bacterial populations in *B. glabrata*. We enumerated the aerobic, heterotrophic colony-forming bacterial populations of 188 individual snails collected from locations on Puerto Rico, St. Lucia, and Guadeloupe, and a further 57 snails from several experimentally manipulated populations (Table 1). The bacterial density

TABLE 1. *Bacterial density in individual B. glabrata snails from field and laboratory populations*[a]

Sample	No. in sample	Snail wt (g)	Bacterial density × 10^6 CFU/g of tissue
Aibonito, P.R.	12	0.11 ± 0.04	10.5 ± 2.1
Humacao, P.R.	11	0.33 ± 0.04	4.4 ± 1.1
Trujillo Alto, P.R.	13	0.07 ± 0.01	31.4 ± 8.0
Mal Pico, P.R.	36	0.10 ± 0.01	14.7 ± 1.5
Rio Piedras, P.R.	29	0.41 ± 0.01	4.6 ± 1.4
Artificial snail pond, Morne Fortune, St. Lucia	10	0.10 ± 0.003	21.0 ± 3.5
Marquis Valley, St. Lucia	20	0.03 ± 0.003	59.6 ± 11.0
Soufriere, St. Lucia	31	0.06 ± 0.003	28.0 ± 4.9
Grande Etang, Guadeloupe	13	0.36 ± 0.03	23.7 ± 4.5
Beaugendre, Guadeloupe	13	0.14 ± 0.02	40.9 ± 6.9
Soufriere, transferred to Cambridge	4	0.06 ± 0.003	165.6
Borricaud, Guadeloupe, aquarium-reared offspring	10	0.07 ± 0.001	237 ± 67
Contaminated axenic P.R. albino snails	9	0.03 ± 0.007	7,500 ± 1,300
Aquarium-reared P.R. albino snails	10	0.09 ± 0.01	114 ± 32
Aquarium-reared P.R. albino snails	12	0.12 ± 0.01	150 ± 47
Aquarium-reared P.R. albino snails	12	0.01 ± 0.001	103 ± 59

[a] All values are means ± standard errors.

within the wild snails was inversely related to tissue weight. In different locations, most of the snails collected tended to fall into specific size classes. In some areas, only quite large or quite small snails were collected. Thus, the bacterial density of snails from the Marquis Valley in St. Lucia was very high, whereas the densities in the large snails from Rio Piedras and Humacao, P. R., were very low. The two Guadeloupe populations showed the same inverse size-density relationships, but their densities were somewhat higher than those in similar-sized snails from Puerto Rico or St. Lucia. Both snail size and bacterial density varied by about one order of magnitude in all wild snails measured. We do not know the reason for this relationship, but it seems likely that young (very small) snails obtain a microflora of a specific size soon after hatching and then maintain their bacterial populations at those numbers as they increase in size. Therefore, the bacterial density decreases as the snails grow.

Snails cultured in laboratory aquaria have higher bacterial densities than those in wild populations. The albino Puerto Rican snail strain has been in culture for perhaps 20 years, and our colonies are over 2 years old. These snails have a higher mean bacterial density than some of our Puerto Rican samples. However, we have found several individual wild snails in Puerto Rico with densities of 70×10^6 to 100×10^6 colony-forming units (CFU)/g.

The snails collected at Borricaud, Guadeloupe, and then cultured in aquaria also had much higher bacterial densities than their wild counterparts. These high densities were attained after only one generation in culture.

While in St. Lucia, we took snails collected at Soufriere and immersed them for 1 day in a stream at Morne Panache. In other immersion experiments run at this location, high snail mortalities have been noted (M. Prentice, Rockefeller Foundation and Research and Control Department, St. Lucia, personal communication). These snails were then sampled for bacterial densities soon after they were returned to Cambridge. The results suggest that higher bacterial densities caused by the stresses accompanying transfer to new habitats can occur relatively quickly.

The final set of snails sampled contained formerly axenic individuals reared in test tubes in the presence of 100 IU of penicillin per ml. During feeding with Formalin-killed *Escherichia coli* or weekly transfer to fresh medium, some of these snails were contaminated from an unknown source. After up to 2 months of further culture, these contaminated snails had exceptionally high bacterial densities, more than 2 orders of magnitude greater than those of wild Puerto Rican snails. Under permissive conditions, *B. glabrata* apparently has the capacity to harbor certain bacterial populations much larger than those usually found in wild snails in nature.

The results of our bacterial enumerations in various *B. glabrata* populations indicate that wild snails maintain bacterial densities lower than those which can be found in snails that have encountered experimental stresses. Transfer to new environments results in increases in bacterial density, and the higher densities are maintained in laboratory-cultured offspring.

The results of our comparisons of water and snail bacterial populations in the laboratory aquaria suggest that the snail bacterial popula-

tions are buffered to some extent from the water populations. Over a 7-week period, the water and snail bacterial densities in the aquarium containing 2- to 7-mm snails were $8.9 \pm 2.9 \times 10^3$ CFU/ml and $1.0 \pm 0.6 \times 10^8$ CFU/g, respectively. In the aquarium with 7- to 18-mm snails, the populations were $1.3 \pm 0.5 \times 10^5$ CFU/ml for water bacteria and $1.4 \pm 0.5 \times 10^8$ CFU/g for snail bacteria. Statistical comparison revealed that whereas the water populations differed significantly ($P < 0.05$), the snail densities were not significantly different ($P < 0.05$). Although it is conceivable that the bacterial densities may be higher in grossly polluted water, our results show that the bacterial populations in the snails do not respond in an extremely sensitive fashion to water populations.

Analysis of the predominant bacterial species in individual *B. glabrata*. We extended our analyses of the bacterial flora of *B. glabrata* to the identification and enumeration of the numerically predominant bacterial types in each snail (Table 2). As a result of incomplete sampling of the Puerto Rican collection, and the death of some isolates after isolation and initial transfer, the results reported below are based on fewer data than the enumerations described above. Table 2 shows the percentage of snails in each collection that had a specific bacterial genus as the predominant colony type on a pair of TGY plates. *Pseudomonas* spp. and *Acinetobacter calcoaceticus* were the most common predominant bacteria in all groups of snails. *Vibrio extorquens*, a red-pigmented organism that occurred only in the wild snails, was the only predominant *Vibrio* species. *V. extorquens* did not grow in the API 20E system. It was identified by using conventional tubed or plated media and the characters listed in Weaver et al. (23). Although this organism is listed in *Bergey's Manual* (3) under the *Methylomonadaceae*, we used the generic designation in the slightly more recent Center for Disease Control publication (23). Likewise, members of the *Enterobacteriaceae* occurred as the predominant colony types only in wild snails. The dominant bacterial types in wild snails were evenly distributed among the *Pseudomonas-Acinetobacter*, *Enterobacteriaceae*, and *Aeromonas-Vibrio* groups (Table 3). These snails also had the most diverse predominating microflora. The Puerto Rican laboratory snails, with a long history in lab culture, had the least diverse microflora, which was composed essentially of *Pseudomonas* and *Acinetobacter*. Snails transferred from the field to laboratory culture appeared to be intermediate between the two groups: members of the *Enterobacteriaceae* were not predominant in any snails, and *Aeromonas* was found to be predominate in a significant portion of the snails sampled.

Table 3 presents information describing the ecology of each predominating genus in the snails. For each predominating genus, two ratios are shown. The first is the mean proportion of colonies of each genus in each snail in which that genus was predominant. For example, if *Pseudomonas* was found to be predominant in three snails and made up 60, 70, and 80% of the flora of each of the snails, the mean percentage of the genus in the total count would be 70%.

For all the genera except *Vibrio* and *Pasteurella*, when a given organism was predominant in a snail, it usually made up 40 to 50% of the total population in that snail. *V. extorquens* was found to be predominant in several snails collected in the field. When *V. extorquens* was the predominant organism in a snail, it made up to 75% of the total microflora. In laboratory snails, a *Pasteurella* sp. was found to play a role similar to that of *V. extorquens* in the wild snails. When predominant, it tended to make up over 70% of the total flora of the snails. These organisms may be competitively superior to other members of the snail microflora so that when they colonize

TABLE 2. *Percentage of snails in each collection with indicated bacterial genus predominant in each snail's microflora*

Location	*Aeromonas*	*Flavobacterium*	*Enterobacter*	*Citrobacter*	*Providencia*	*Vibrio*	*Acinetobacter*	*Pseudomonas*	Other
Puerto Rico	—[a]	—	09	—	26	24	06	36	—
St. Lucia	03	—	13	20	—	—	03	30	27[b]
Guadeloupe	36	—	09	—	—	36	09	09	—
Lab, P.R.[c]	18	06	—	04	—	—	14	46	10[d]
Contaminated lab, P.R.	—	22	—	—	—	—	11	66	—
Lab, Guadeloupe	—	—	—	—	—	—	60	40	—
Transferred, St. Lucia	23	—	—	—	—	—	38	31	—

[a] —, Not predominant in any of sampled snails.

[b] Gram positives, *Bacillus* spp.

[c] Snail colonies in laboratory aquaria derived from Puerto Rico and Guadeloupe. See text (sampling from aquarium snail colonies) for explanation.

[d] *Pasteurella* sp.

TABLE 3. *Predominance of bacterial genera in typically and atypically colonized B. glabrata snails*

Predominating genus	Mean % of CFU in snails[a]	Colonization ratio[b]
Pseudomonas	37.9 ± 2.6	0.87 ± 0.14
Providencia rettgeri	60 ± 16.3	1.22 ± 0.45
Vibrio extorquens	74.8 ± 7.9	2.05 ± 0.96
Acinetobacter	51 ± 5.2	0.91 ± 0.26
Enterobacter	60 ± 15.8	0.86 ± 0.20
Aeromonas hydrophila	48 ± 5.4	0.65 ± 0.13
Citrobacter freundii	50 ± 8.3	0.90 ± 0.32
Flavobacterium	25.7 ± 9.2	0.92 ± 0.48
Pasteurella	70.8 ± 13.7	2.52 ± 1.06
Gram positive organisms	53 ± 5.5	0.72 ± 0.19

[a] The fraction of the total CFU in snails contributed by each genus or species when that organism is the predominant colony type in a snail. See also discussion of analysis of predominant bacterial species in Results.

[b] The density of bacteria in an individual snail divided by the mean density for all snails in the sample from which the snail was taken. The figures are the average colonization ratios for all snails in which each species was the predominant organism. See discussion in Results for further explanation.

a snail, they tend to make up a greater proportion of that snail's bacterial flora than other, more frequent predominating organisms.

Additional information on the patterns of colonization of snails by their predominant bacterial inhabitants was acquired by computing the ratio of the individual bacterial density in each snail to the mean bacterial density for that snail's population. For example, a snail with a density of 10^7 CFU/g from a snail population in which the mean bacterial density was 1.5×10^7 CFU/g would have a ratio of 1.5. If the *Pseudomonas*-dominated snails mentioned above had ratios of 1.0, 1.1, and 1.2, the mean colonization ratio would be 1.1. This ratio is a convenient index of the degree of bacterial colonization in any given snail. We have found that some snails have bacterial densities much greater than the mean density for snails in their population. Table 3 also shows the mean colonization ratio for snails with each predominant organism. Most of the predominating genera were found in normally colonized snails (i.e., those with ratios near 1). However, snails in which *V. extorquens* or *Pasteurella* sp. were numerically predominant usually had bacterial densities greater than the average for snails in their particular sample. These organisms not only make up a greater fraction of the total microflora in snails in which they are the most numerous inhabitants, but also predominate in snails which have an unusually dense bacterial bioburden.

DISCUSSION

There are few reports concerning the bacteriology of *B. glabrata* or other aquatic snails. Most investigations of which we are aware concern the identification or isolation of human or snail pathogens or unusual bacteria (1, 2, 7-9, 16, 19, 20). This investigation is the first systematic analysis of the microbial populations in freshwater snails.

Our data indicate that bacterial densities differ from one snail population to the next, and that densities appear to be inversely related to snail size. The findings also show that our aquarium populations have higher bacterial densities than the field populations, even for recently established laboratory colonies. We do not know the reasons for these differences, but several explanations seem reasonable.

Snails in the field eat the leaves of submerged vegetation. The abundance and quality of the food source in different habitats could influence the bacterial populations of the gut. The bacterial populations in water could influence the bacterial flora of the external surfaces and mantle cavity of each snail. However, our data on water and snail bacterial populations do not show any striking influences of this type. More research on the relationships between snail, food, and water bacteria is needed to clarify these points.

The differences between densities in field and laboratory snails could be explained by differences in food, water, and snail population densities among these habitats. The snail food and water in the laboratory seem cleaner than in the field, but we do not have data to back up these impressions.

We found that about 10% of the snails in each sample had bacterial densities more than twice as great as the mean density for the sample from which they came. In all cases, these density values fell outside the upper 99% confidence limit for snail bacterial density. We regard these snails as being atypically colonized by large numbers of bacteria. There are tendencies for these high-bacterial-density snails to have different predominant bacterial species than the majority of snails (Table 3). In the field, the bacterial flora of such snails is composed of 70% *V. extorquens*. In the laboratory, high-bacterial-density snails have a microflora consisting of 70% *Pasteurella*. The other genera found to be predominant rarely make up more than 70% of a snail microflora and are rarely predominant in high-density snails with colonization ratios of over 2. The consistent finding of *V. extorquens* and *Pasteurella* sp. as the major inhabitants of high-density snails suggests that the presence of these organisms may be used to signify atypically colonized snails. We believe that snails with these organisms predominating, and with colo-

nization ratios of 2.0 or greater, are microbiologically compromised snails.

There is abundant evidence that microbiologically compromised macroorganisms exist in nature and that these often harbor organisms pathogenic to the species in question. Much of this material has been reviewed recently (17). High bacterial populations in the disadvantaged individuals appear to be useful indicators of the compromised state (11, 14, 18).

Results reported here provide further insights into the nature of normal and altered microfloras of healthy and compromised snails. Snails subjected to the stresses of transport and environmental change had increased bacterial populations. Such transitional snails also had a higher incidence of *Aeromonas hydrophila*, a known snail pathogen (9).

We found that relatively few aerobic heterotrophic genera were preponderant in wild and laboratory snails. Gram-positive organisms do occur in *B. glabrata*, but seldom predominate. Field snail populations have a higher diversity of these dominant genera. *Enterobacteriaceae* are predominant in about one-third of all field snails, but are apparently outcompeted or selected against when these snails are transported into the laboratory. Similarly, *V. extorquens* has never been isolated from a snail in our laboratory colonies. The microflora of *B. glabrata* appears to be somewhat plastic. Possibly, variations in diet, habitat, and environmental conditions experienced by different snails in nature permit different bacteria to predominate in the snail microflora. When snails are moved into laboratory culture, the microflora is impoverished in diversity and increased in numbers.

Our data show that sick snails, which are difficult if not impossible to distinguish by inspection, can be recognized by the microbiological analyses we have described. This finding, and the discovery of several recognized opportunistic pathogens in the snails, has great relevance for biocontrol of these vector snails. Microbiologically compromised snails may harbor opportunistic pathogens that could be used as microbiological agents for snail control.

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