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LACK OF SURFACE-ASSOCIATED MICROORGANISMS IN A MIXED SPECIES COMMUNITY OF FRESHWATER UNIONIDAE†

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ABSTRACT To determine whether unionids contain surface-attached endosymbiotic bacteria, ciliates, or fungi, we used scanning electron microscopy to examine the epithelial surface of various organs within the digestive system and mantle cavity of temperate river and lake unionids on a seasonal basis. We also cultured material removed from the lumen of these same organs and from the mantle cavity to detect cellobiose-, cellulose- and chitin- degrading microbes. No true endosymbiotic fauna were observed attached to the surface of the digestive or mantle tissues of any species of unionid. Microbial growth on cellulose or chitin bacteriological media varied by season and habitat, but not by unionid species or source of the isolate. Lake unionids did not contain microbes capable of digesting cellulose or chitin, whereas unionids from the river site did in March and August, but not in December. Since these cultured cellulose- and chitin-degrading bacteria were never found attached to any unionid tissues, they appeared to be transient forms, not true endosymbionts. Microbes capable of digesting cellobiose were found in every unionid collected in all seasons and habitats, but again, no microbes were directly observed attached to unionid tissues. If unionids, like most other invertebrates, lack digestive enzymes required to initiate primary bond breakage, then the lack of cellulolytic and chitinolytic endosymbionts would affect the ability to utilize cellulose or chitin foods. Thus, in captivity dry feeds based on corn, soybeans, or nauplii should be pre-digested to ensure maximum absorption of nutrients by unionids. The lack of cellulolytic or chitinolytic endosymbionts should not affect relocation success, though the seasonal role of transient microbes in unionid nutrition requires further investigation.

KEY WORDS: gut microflora, endemic microbes, Unionidae

INTRODUCTION

Freshwater unionids in North America are being extirpated at alarming rates due to factors such as habitat degradation and competition from the exotic zebra mussel (*Dreissena polymorpha* Pallas) (Williams et al. 1993). At this time, conservation efforts for adult unionids include relocation into new habitats and intensive aquaculture, but mortality rates have been high with most adults surviving less than three years (Cope & Waller 1995; Lellis and Johnson 1998; Gatenby et al. 1999). Although these high mortality rates are undoubtedly due to a host of different factors, one problem area has been identification of dietary requirements. Little information exists on dietary preferences and required nutrients necessary for formulating a captive diet, and evaluating food resources in new habitats or refugia. The few dietary studies in natural habitats have shown that unionids ingest a wide variety of potential food items including algae, detritus, fungus, rotifers, and zooplankton, with detritus as the dominant component in both the mantle cavity and gut lumen (Lefevre & Curtis 1910; Jiffry 1984; McMahon 1991; Nichols & Garling 2000).

The ingestion of large amounts of detritus implies that detritus is actively selected and thus may play a significant dietary role. This role is not easy to characterize as detritus in aquatic systems represents a complex matrix often containing cellulose from aquatic and terrestrial sources, chitin from fungus, rotifers, and zooplankton, and which has in turn been colonized by bacterial, fungal, and protistan fauna. Thus, ingested detritus could function merely as a substrate for the preferred food item, the associated epiorganisms, or could function as a direct source of nutrients obtained from cellulose or chitin degradation. One complicating factor is that cellulose and chitin are highly complex polysaccharides that require specific enzymes to initiate primary bond breakage. Such primary enzymes are rarely produced endogenously in aquatic invertebrates, and are recorded for very few marine bivalves, although cellulases in general have been detected in both marine and freshwater mollusks. In unionids, the concentration of cellulases present has actually proven to be a successful technique for assessing the health status of the animal (Haag et al. 1993; Farris et al. 1994). However, these cellulase concentrations were obtained from total animal bioassays, from unionids freshly removed from the field, and were not further identified to type. It has not been determined if primary cellulases were present, and if so, whether they were produced endogenously by the unionid, or exogenously by the microbial community associated with ingested food items. Furthermore, chitinases of any type have not yet been reported for either freshwater or marine bivalves.

Most aquatic animals that feed directly on detrital cellulose access the necessary primary cellulases through a symbiotic relationship with some type of bacteria/microbe, and contain recognizable endosymbiotic fauna somewhere in their digestive tracts (black fly larvae, Taylor et al. 1995; crane fly larvae, Klug & Kotarski 1980; mullet, Mountfort & Rhodes 1991). Microbial and endosymbiont/nutritional relationships among bivalves are far more complex than those reported for other aquatic invertebrates and often vary by species as well as by location. True endosymbiotic relationships range from the consistent obligate communities of microbes directly buried within gill tissues of deep-sea hydrothermal vent bivalves (Wood & Kelly 1989), to spirochaetes merely attached to the outside epithelial layer of digestive tissue or to the crystalline style (Bernard 1970; Conway & Capuzzo 1989; Prieur et al. 1990). Geographical variability is common, with endosymbionts existing inside some species at some locations, but not in others (Bernard 1970), while some marine bivalves never exhibit endosymbiotic relationships (Garland et al. 1982). Studies on freshwater bivalve- microbial relationships are very limited (Starliper et al. 1997) and have focused mainly on bacterial communities found in the gut lumen or passing through the intestinal tract. As in marine bivalves (Prieur et al. 1990; Harris et al. 1998),

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microbes found on the epithelial surface of various mantle and digestive organs (foot, gills, labial palps, stomach, ciliary tufts, crystalline style and style sac, digestive gland, and fore-mid-hind intestinal tract) except in one animal (Fig. 1a-d). All epithelial layers of these tissues were covered in March and August with a heavy layer of mucus. Food particles, including bacteria, were often seen trapped in this mucus layer (Fig. 1e and f), but no attached bacteria or other microbes were associated with this mucus layer or below it on the surface of the epithelium itself. The one exception was a fungal mat growing on the mantle tissue, at the base of the excurrent siphon, collected from one *L. siliquoidea* in August (Fig. 2).

Even though no obvious attached microbes were seen, every unionid examined, from all sites, contained what appear to be attached round structures, possibly spores, in their intestinal tracts. These structures were always found below the mucus layer and between the enterocytes comprising the intestinal wall (Figs. 3 and 4). They were large, about 1 μ in diameter, and were attached by a stalk to the unionid tissue (Fig. 4d and f). Though consistently seen, attempts to rear these spores (if that is what they were) in isolation, for further elucidation, failed.

Microbial Growth on Bacteriological Media

Microbial growth varied by locality, media type, and season but not by unionid species, replicate, organ source, or the presence or absence of oxygen. All (100% of the 336 samples) of the samples from the Huron River population collected in March and August

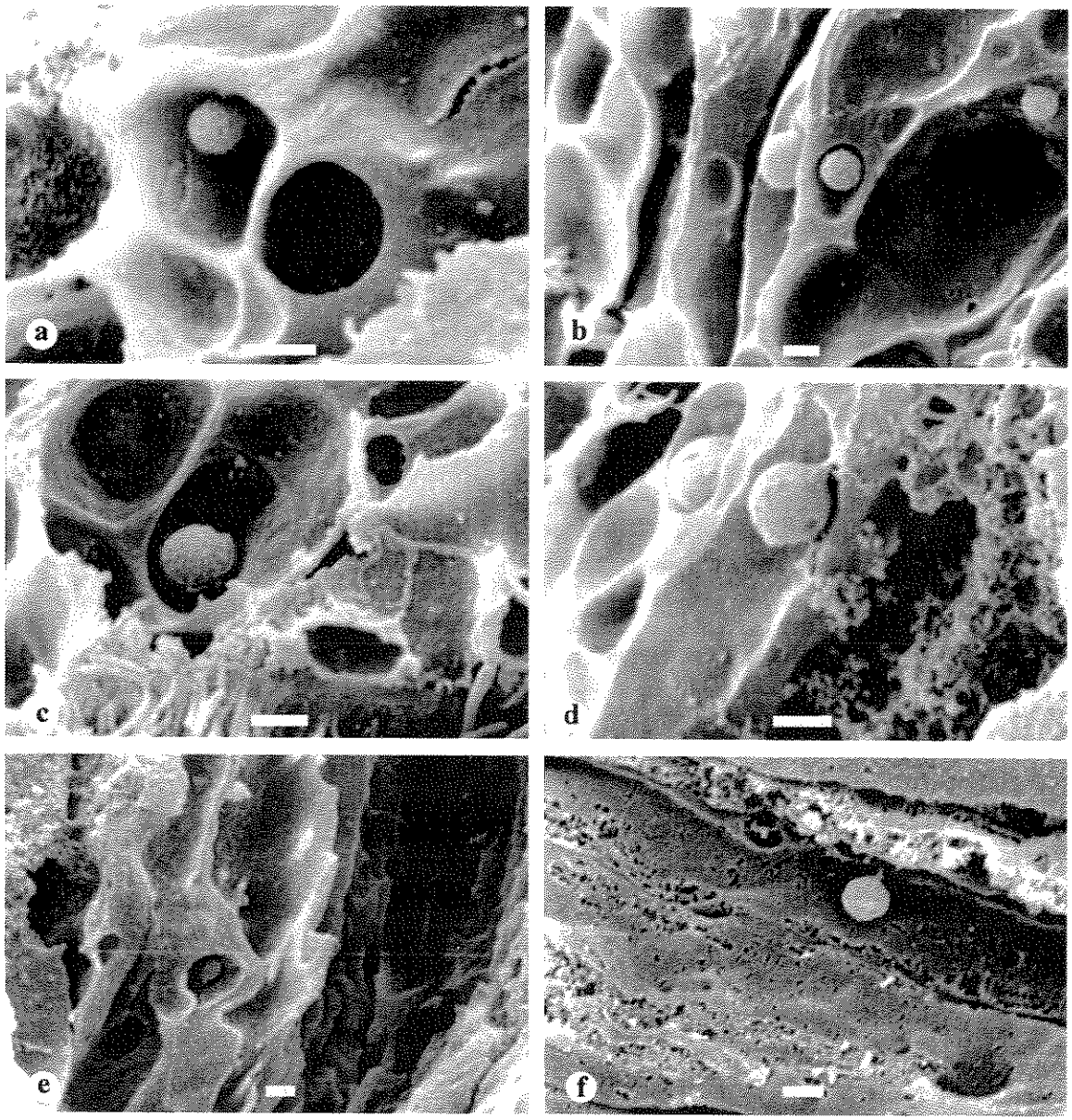

Figure 4. Scanning electron micrographs of the unidentified attached spore-like structures found in the intestinal tract of various species of freshwater unionids collected from several sites in southeast Michigan: (a) *Pyganodon grandis*, Huron River; (b) *Lampsilis siliquoidea*, Huron River; (c) *Lampsilis ventricosa*, Huron River; (d) *Ptychobranchus fasciolaris*, Huron River; (e) *Elliptio dilatata*, Vineyard Lake; and (f) Pyganodon *grandis*, Four Mile Lake. Scale bars = 1μm (a–e).

contained cellulose-, chitin-, and cellobiose-degrading bacteria, regardless of unionid species, organ from which the inoculant was obtained, or whether the sample was grown aerobically or anaerobically (Table 1). In all 336 samples, bacterial growth was noted within 12 h of inoculation based on gas production and clouding of the media. The bacterial communities developing in the cellulose and chitin media were similar in that all were motile, were fermenters, and facultative anaerobes (Fig. 5), but they differed in size. The rod-shaped bacteria growing in the chitin were longer (avg. 3.96 μm) than those in the cellulose media (avg. 2.26 μm). The bacterial community that grew in the cellobiose was dominated by non-motile cocci, which were also fermenters and facultative anaerobes. Rod-shaped bacteria were present, but even smaller in length (avg. 1.95 μm) than those found in the other types of media. However, in the December samples, none of the 112 samples inoculated in either cellulose or chitin showed any growth up to 120 hours. In contrast, the December cellobiose samples ($n = 56$) did show a 100% response to this media within 12 h, regardless of unionid species, organ source, or whether the sample was grown aerobically or anaerobically.

The lake samples, *P. grandis* from Four Mile and *E. dilatata* from Vineyard, differed from Huron River unionids in that no microbial growth occurred in either cellulose or chitin media regardless of season, or organ source (total of 168 samples for both lakes) (Table 1). However, like the Huron River animals, these lake unionids consistently showed activity in 100% of the 84 cellobiose samples, regardless of season, lake, or organ source. As with the Huron River unionid cellobiose samples, the bacterial community growing on the cellobiose media was dominated by non-motile cocci that were fermenters and facultative anaerobes. In size and appearance they were identical to those observed in the Huron River unionid samples.

DISCUSSION

None of the unionids samples examined contained true endemic microflora. In order to be considered a true endosymbiont,

TABLE 1.

Growth of microorganisms removed from the lumen of mantle cavity (mantle) and digestive tract organs (gut) on cellulose, cellobiose, and chitin bacteriological media, under aerobic and anaerobic (in parenthesis) conditions.

Season	Species	Cellulose		Chitin		Cellobiose	
March	*Lampsilis ventricosa*	mantle	+(+)	mantle	+(+)	mantle	+(+)
	Huron River	gut	+(+)	gut	+(+)	gut	+(+)
and	*Lampsilis siliqouidea*	mantle	+(+)	mantle	+(+)	mantle	+(+)
	Huron River	gut	+(+)	gut	+(+)	gut	+(+)
Aug.	*Ptychobranchus fasciolaris*	mantle	+(+)	mantle	+(+)	mantle	+(+)
	Huron River	gut	+(+)	gut	+(+)	gut	+(+)
	Pyganodon grandis	mantle	+(+)	mantle	+(+)	mantle	+(+)
	Huron River	gut	+(+)	gut	+(+)	gut	+(+)
	Pyganodon grandis	mantle	0(0)	mantle	0(0)	mantle	+(+)
	Four Mile Lake	gut	0(0)	gut	0(0)	gut	+(+)
	Elliptio dilatata	mantle	0(0)	mantle	0(0)	mantle	+(+)
	Vineyard Lake	gut	0(0)	gut	0(0)	gut	+(+)
Dec.	*Lampsilis ventricosa*	mantle	0(0)	mantle	0(0)	mantle	+(+)
	Huron River	gut	0(0)	gut	0(0)	gut	+(+)
	Lampsilis siliquoidea	mantle	0(0)	mantle	0(0)	mantle	+(+)
	Huron River	gut	0(0)	gut	0(0)	gut	+(+)
	Ptychobranchus fasciolaris	mantle	0(0)	mantle	0(0)	mantle	+(+)
	Huron River	gut	0(0)	gut	0(0)	gut	+(+)
	Pyganodon grandis	mantle	0(0)	mantle	0(0)	mantle	+(+)
	Huron River	gut	0(0)	gut	0(0)	gut	+(+)
	Elliptio dilatata	mantle	0(0)	mantle	0(0)	mantle	+(+)
	Vineyard Lake	gut	0(0)	gut	0(0)	gut	+(+)
	Pyganodon grandis	mantle	0(0)	mantle	0(0)	mantle	+(+)
	Four Mile Lake	gut	0(0)	gut	0(0)	gut	+(+)

Response code: + indicates bacterial growth and media degradation occurred within 12 hours of culture; 0 indicates no bacterial growth seen from 12–96 hours after inoculation. Sample size differs and is discussed in the methods section of text.

Figure 5. Scanning electron micrograph of the bacterial community cultured from the mantle cavity and gut content of freshwater Unionidae. (a) shows the bacterial community that developed in cellulose media, while (b) shows the community that developed in the chitin media. Scale bar = 1 μm.

microbial fauna must demonstrate certain characteristics which include: at least part of the endosymbiont community must be physically attached to the host's body tissues to ensure retention inside the animal; endosymbiont densities should be high ($>10^{10}$/g), and; the endosymbiont must be consistently found inside the host regardless of season or location (Yokoyama and Johnson 1993; Taylor et al. 1995). These criteria were not met. One problematic question remains regarding the unidentified spore-like structures attached or associated with every unionid intestinal tract we examined. Our inability to culture or isolate enough of these structures for further identification prevents us from labeling them as endosymbionts or to hypothesize their relationship with the unionid host. While in appearance and size these structures resemble the fruiting bodies, or zoospores, produced by fungus or a bacterium, a hyphal vegetative phase was only found once and in the mantle, not gut. The one exception, the fungal hyphae, attached to a specimen of *L. siliquoidea* (Fig. 2) and lacked spores or fruiting bodies. Even if these attached structures are spores, their limited numbers are not consistent with endosymbiont community criteria, and likely represent remnants of transient fauna. Fungi are a common component of the planktonic material drifting in the water of both Four Mile Lake and the Huron River (Nichols and Garling 2000). Occasional microbial spores are not uncommon in the intestinal tracts of some marine bivalves (Kueh and Chan 1985).

The presence of copious amounts of mucus in the mantle cavity and gut lumen of unionids (Fig. 1e) may mask or prevent the colonization of tissues by endosymbionts. Hyphal structures of fungi could easily be hidden in thick mucus layers. While surface-associated microsymbionts including fungi have been identified in many animals including fish (Mountfort and Rhodes 1991) and ruminants (Lowe et al. 1987; Sijtsma and Tan 1993) using the same techniques we used in this study, none of these vertebrates produce and utilize mucus in food handling and processing as do unionids. However, during December, when concurrent studies in the Huron River indicated unionids were not feeding (Nichols and Garling 2000), very little mucus was present inside the animals, and yet no attached microflora could be observed. A true endosymbiotic community would still have remnant, attached fauna, even when feeding was not occurring. Garland et al. (1982) have hypothesized that in oysters heavy mucus production prevents endosymbiont attachment on tissues. Such mucus hindrance might also limit the development of surface-attached endosymbiotic communities in unionids as well.

The variability of the results of attempted culture of microorganisms on cellulose or chitin media support our conclusion that unionids lack true endosymbiotic microbes capable of digesting these substrates. Microbial growth occurred on cellulose and chitin during periods that concurrent studies in the Huron River indicated the unionids were feeding (late February to late November, Nichols and Garling 2000). Similarly, microbial growth on these culture media did not occur during December when the unionids in this river were not feeding. If endosymbionts were present, we should have been able to culture them in December samples. The differences in microbial response to culture media, in combination with the fact that no microbes were found attached inside the unionids, indicate that cellulolytic and chitinolytic microbial communities represent transient fauna and not endosymbionts. The total lack of microbial growth in cellulose and chitin media by the Four Mile and Vineyard lake samples is surprising. Likely, this may correspond to significant differences in lake versus river microbial communities.

The ability to culture a microbial community on the cellobiose media from all unionids, regardless of species, season, or habitat, is certainly a trait associated with endemic microbial fauna. However, since no microbes were found attached to any unionid internal tissues, we have concluded that these starch-degrading microbes were transient fauna and not endosymbionts. While the spore-like structures found attached between the enterocytes of all unionid intestinal tracts could hypothetically be related to this cellobiose community, the lack of further identification of these structures prevents making this direct association.

Assuming primary cellulases and chitinases are not endogenously produced, the lack of cellulolytic or chitinolytic endosymbionts in unionids means that initial bond breakage must rely on transient microbial fauna. A reliance on such transient microbes could influence captive maintenance success if dry diets based on cellulose (corn/soybean) or chitin (nauplii) are fed to unionids. Efficient utilization of these feeds by these bivalves would require prior microbial predigestion in order to provide the necessary initial bond breakage to break these complex polysaccharides.

Unionids appear unique among aquatic invertebrates in that they consume detritus and yet lack microbial endosymbionts that would aid in digesting complex polysaccharides. While this may imply that detritus merely serves as a convenient substrate for preferred food items, i.e., attached epimicroorganisms, further work is needed to fully identify digestive enzymes produced by

unionids. Dependence of unionids on microbes appears complex and combines elements of selective predation, subsequent enhancement of non-prey species, and a reliance on transient fauna. Our observations do not support the endosymbiont model characteristic of well-known vertebrate/microbe associations. The role of transient microbial fauna, particularly the importance of different microbial species assemblages, may very well be key factors influencing unionid survival in relocation and aquaculture efforts.

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