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Surface Morphology of *Saccinobaculus* (Oxymonadida): Implications for Character Evolution and Function in Oxymonads

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Examination of surface morphology of the oxymonad genus *Saccinobaculus* from the gut of the wood-feeding cockroach *Cryptocercus punctulatus* with scanning and transmission electron microscopy reveals several new characters not observable with light microscopy. These include small concavities covering the external surface, a glycocalyx, coated pinocytotic vesicles, and, in one species, unidentified, membrane-bounded organelles with a granular matrix that may represent peroxisomal or mitochondrial derivatives. Unlike representatives of some other oxymonad families, *Saccinobaculus* lacks extracellular surface structures, a holdfast, and, generally, ectobiotic bacteria. We examined the evolution of these and other characters in light of previously published phylogenies of oxymonads based on molecular data. The presence of concavities in *Saccinobaculus* and families Pyrsonymphidae and Oxymonadidae strengthens support for a clade comprising these three families. A glycocalyx appears to be a synapomorphy of all oxymonads, and the presence of ectobiotic bacteria also appears to be ancestral to oxymonads, but lost in *Saccinobaculus*. A holdfast appears to have arisen multiple times. We hypothesize that concavities may play a role in a two-step mechanism for the accumulation and internalization of specific solutes, and that the highly motile and morphologically plastic nature of *Saccinobaculus* cells limits the possibility of retaining a covering of ectobiotic bacteria.

Keywords: *Cryptocercus punctulatus*; glycocalyx; mitochondria; oxymonads; *saccinobaculus*; surface morphology.

Introduction

Oxymonads (order Oxymonadida) are anaerobic, amitochondriate flagellates that live as obligate symbionts of animals, and are united by a suite of distinctive cytoskeletal and ultrastructural features (see Brugerolle and Lee 2000; Simpson et al. 2002 for a review), as well as molecular data (Moriya et al. 2003). Among their more notable features are a lack of mitochondria (or any recognized derivative organelles such as hydrogenosomes or mitosomes), peroxisomes, and Golgi bodies, and the use of a non-canonical genetic code in some members. Their resistance to cultivation, poorly understood nutritional modes and life cycles, and

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the lack of strong evidence for a close relationship to any other major clade of eukaryotes represent some of the major gaps in our knowledge of this group. There are approximately 70 described species of oxymonads in five families, four of which are found exclusively in the hindgut of wood-eating insects such as lower termites and the cockroach *Cryptocercus* (Brugerolle and Lee 2000), where their ecological role remains uncertain. Some recent studies place oxymonads in a large eukaryotic supergroup called Excavata, which includes many other amitochondriate (as well as mitochondriate) protist clades such as euglenids, parabasalids, and diplomonads (Cavalier-Smith 2002; Simpson et al. 2006).

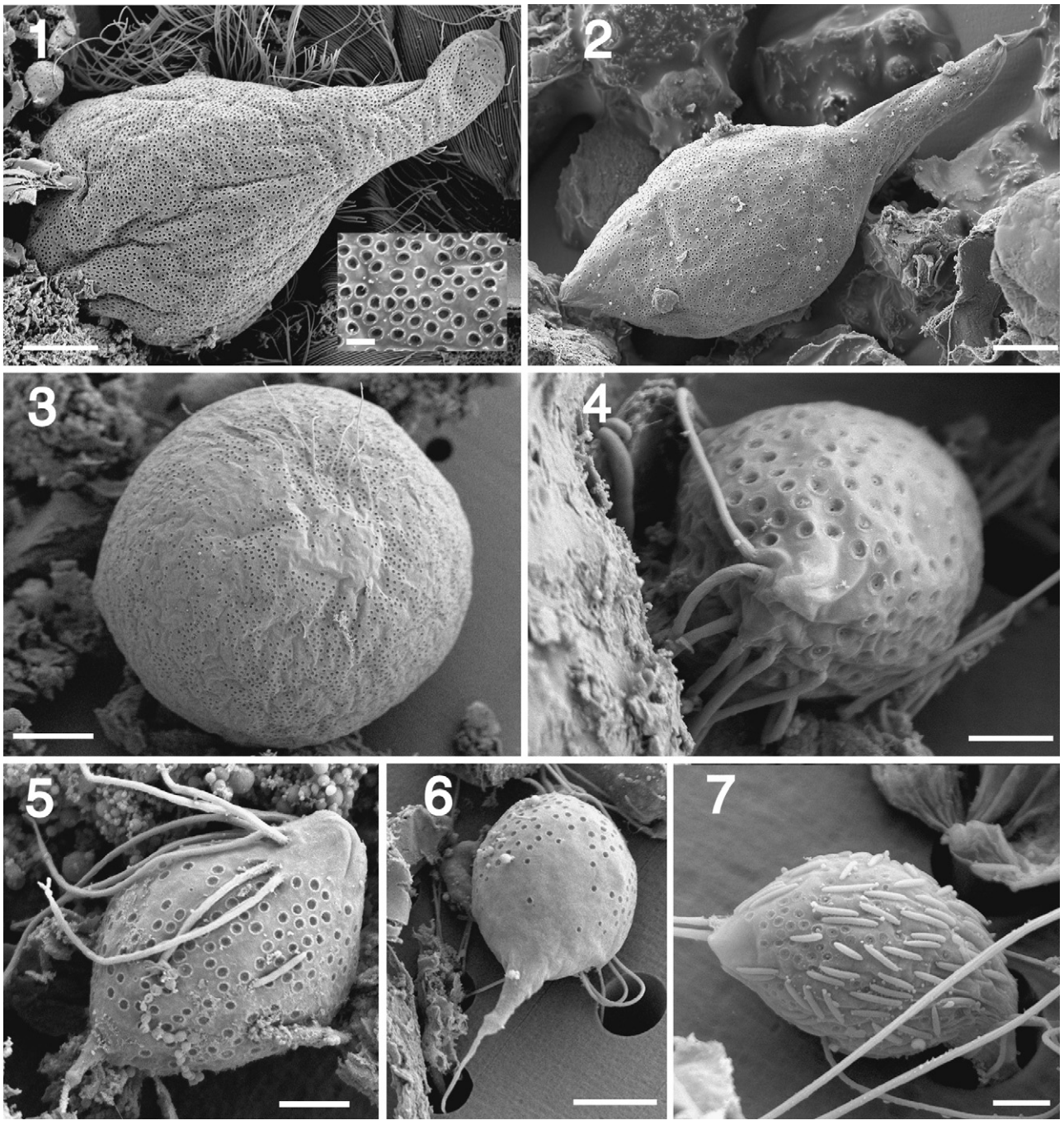
The oxymonad genus *Saccinobaculus*, of the family Saccinobaculidae, is among the more spectacular oxymonads in terms of its behavior and mode of locomotion. Cleveland et al. (1934) originally described three species of *Saccinobaculus* from the hindgut of wood-feeding cockroach *Cryptocercus punctulatus*, still the only known habitat of this genus and family. Each of the three species may bear four, eight, or 12 flagella, but these seem to contribute little to locomotion. The predominant motion in *Saccinobaculus* is effected by its axostyle, a ribbon-like bundle of microtubules running the length of the cell, which undulates at varying amplitudes and frequencies, sometimes throughout its length, and other times restricted to one portion. Movements of the axostyle are often rapid and violent, causing equally rapid and drastic changes in cell shape, ranging from elongate and straight, to bent, to spherical and intermediate forms (see Heiss and Keeling [2006] for a series of light micrographs illustrating this motion). This led Cleveland to compare the motion of *Saccinobaculus* to that of a snake thrashing around in a bag — with the snake representing the axostyle, and the bag the cell — a comparison from which the formal name derives (Cleveland et al. 1934). Due to its resistance to cultivation and extreme morphological plasticity, Cleveland found it very difficult to determine how many species were represented by the diversity of *Saccinobaculus* forms he observed, but ultimately decided on three, based on size and features such as the shape of the axostyle, shape and position of the nucleus, and features relating to nuclear division and reproduction. These species are: *Saccinobaculus minor*, the smallest at 14—30 μm; *S. ambloaxostylus*, at 65—110 μm; and *S. doroaxostylus*, at 150—170 μm (Cleveland et al. 1934). Subsequent investigation of the phylogenetic position of *Saccinobaculus* among oxymonads using molecular data (Heiss and Keeling 2006) found a great diversity of closely related sequence-types, and together these were all weakly -allied with the oxymonad families Pyrsonymphidae and Oxymonadidae.

After its original description based on light microscopy, examination of *Saccinobaculus* with transmission electron microscopy (TEM) followed, focusing mostly on the structure of the axostyle (Grimstone and Cleveland 1965; Hollande and Carruette-Valentin 1970; McIntosh 1973). Unlike families Pyrsonymphidae, Oxymonadidae, and Streblomastigidae (Bloodgood 1974; Leander and Keeling 2004; Maaß and Radek 2006; Rother et al. 1999; Smith et al. 1975; Smith and Arnott 1973), surface morphology of Saccinobaculidae remains unexamined with scanning electron microscopy (SEM), and largely untouched with TEM. Even studies that do present TEM micrographs showing surface structures in *Saccinobaculus* (Grimstone and Cleveland 1965) make only passing reference to them. This is unfortunate, because studies of the surface morphology of other oxymonad families using SEM and TEM have uncovered numerous characters that are not observable with light microscopy (e.g., extracellular surface structures, surface concavities, glycocalyx, pinocytotic vesicles, and ectobiotic bacterial attachment sites), which provide important insights to function and phylogenetic relationships within the group (Bloodgood 1974; Leander and Keeling 2004; Maaß and Radek 2006; Rother et al. 1999; Smith et al. 1975; Smith and Arnott 1973).

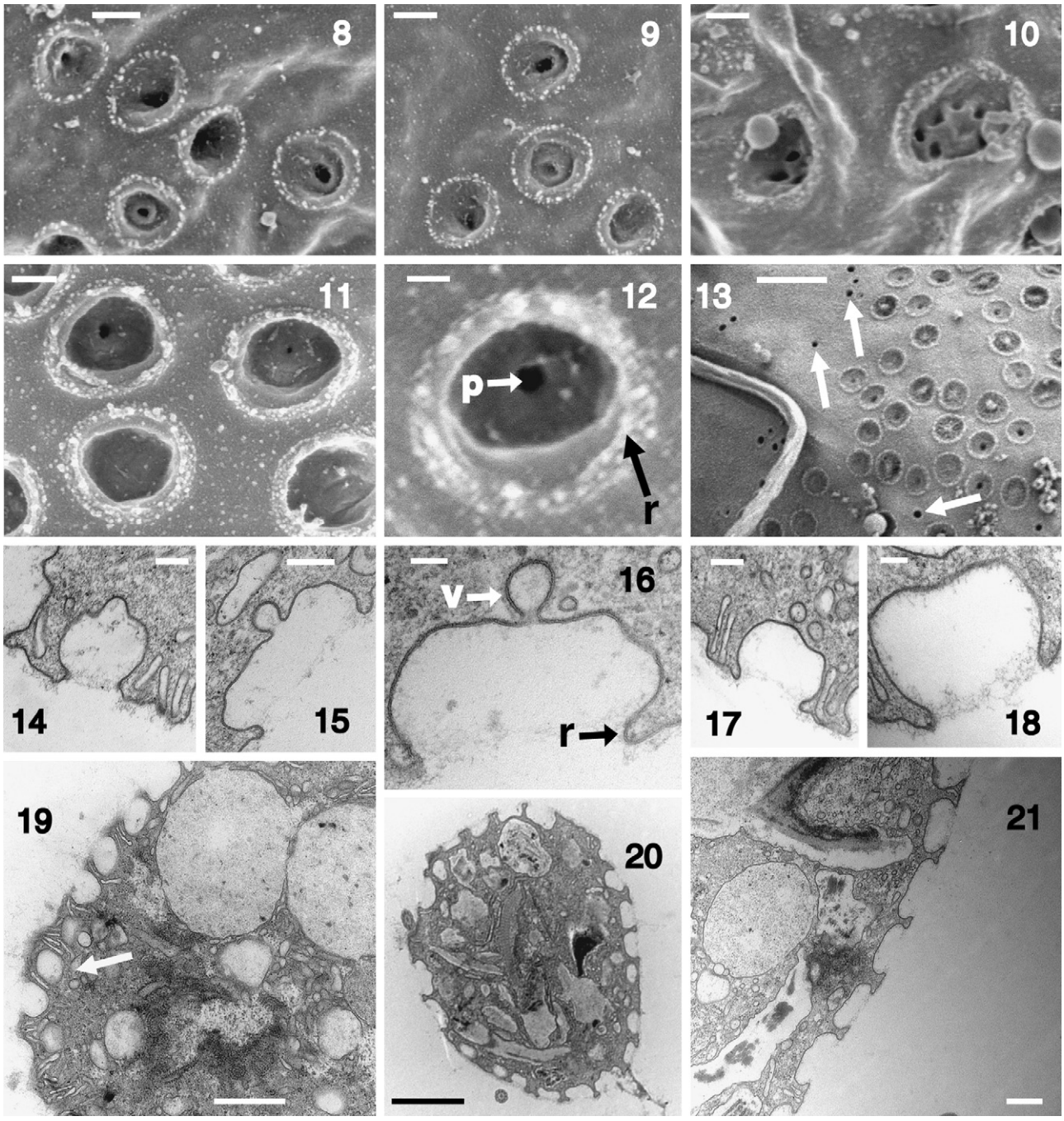
In this study, we seek to complement the existing data on surface morphology of other oxymonad families by investigating this suite of characters in *Saccinobaculus* using both SEM and TEM. We examine hypotheses for the evolution of these characters in oxymonads (within a phylogenetic framework based on molecular studies), as well as their possible functional roles.

Results

Members of the genus *Saccinobaculus* were observed in all individuals of *C. punctulatus* examined, but the abundances of the individual species varied considerably. While cells matching the original description of *S. minor* and *S. ambloaxostylus* (Cleveland et al. 1934) were present and common in all individuals (Figs 1—21), the largest species, *S. doroaxostylus* (Cleveland et al. 1934),



Figures 1—7. SEM micrographs of whole cells of *Saccinobaculus ambloaxostylus* and *Saccinobaculus minor*. **1.** and **2.** Side views of elongate forms of *S. ambloaxostylus* showing a covering of closely spaced concavities (Fig. 1 inset scale bar = 1 μm). The anterior end of each cell is adjacent to the lower left corner in each micrograph (Scale bars = 10 μm). **3.** Anterior view of a spheroidal form of *S. ambloaxostylus* showing flagella near the center of the cell (Scale bar = 10 μm). **4.** Anterior view of *S. minor* (Scale bar = 2 μm). **5.** Side view of *S. minor* showing axostyle protruding from posterior end at lower left (Scale bar = 2 μm). **6.** Side-oblique view of *S. minor* showing axostyle protruding from posterior end at lower left. Note the uneven distribution of concavities (Scale bar = 5 μm). **7.** Side view of an *S. minor* cell with rod-shaped bacterial ectobionts (Scale bar = 2 μm).



Figures 8—21. SEM and TEM micrographs of surface concavities of *Saccinobaculus minor* and *Saccinobaculus ambloaxostylus*. **8.** and **9.** *S. minor* (Scale bars = 200 nm). **10.** *S. ambloaxostylus*. Note multiple pores in the center of each concavity. These are less common than those with only one pore (Scale bars = 200 nm). **11.** and **12.** *S. ambloaxostylus*. Note the raised circular cytoplasmic rim (r) and central pore (p) of each concavity (Scale bars: Fig. 11 = 200 nm; Fig. 12 = 100 nm). **13.** Cell surface of *S. minor* showing small pores (arrows) occurring alongside concavities (Scale bar = 1 μm). **14—18.** Sections through concavities of either *S. ambloaxostylus* or *S. minor*. Note rims (r) and pits or vesicles (v); the pits correspond to the pores seen in surface view (SEM). (Scale bars: Figs 14, 15, 17 = 200 nm; Figs 16, 18 = 100 nm) **19—21.** Sections through *S. minor* or *S. ambloaxostylus* cells showing concavities. Note concavities that appear closed to the outside (e.g., at arrow). (Scale bars: = Fig. 19 = 1 μm; Fig. 20 = 2 μm; Fig. 21 = 500 nm).

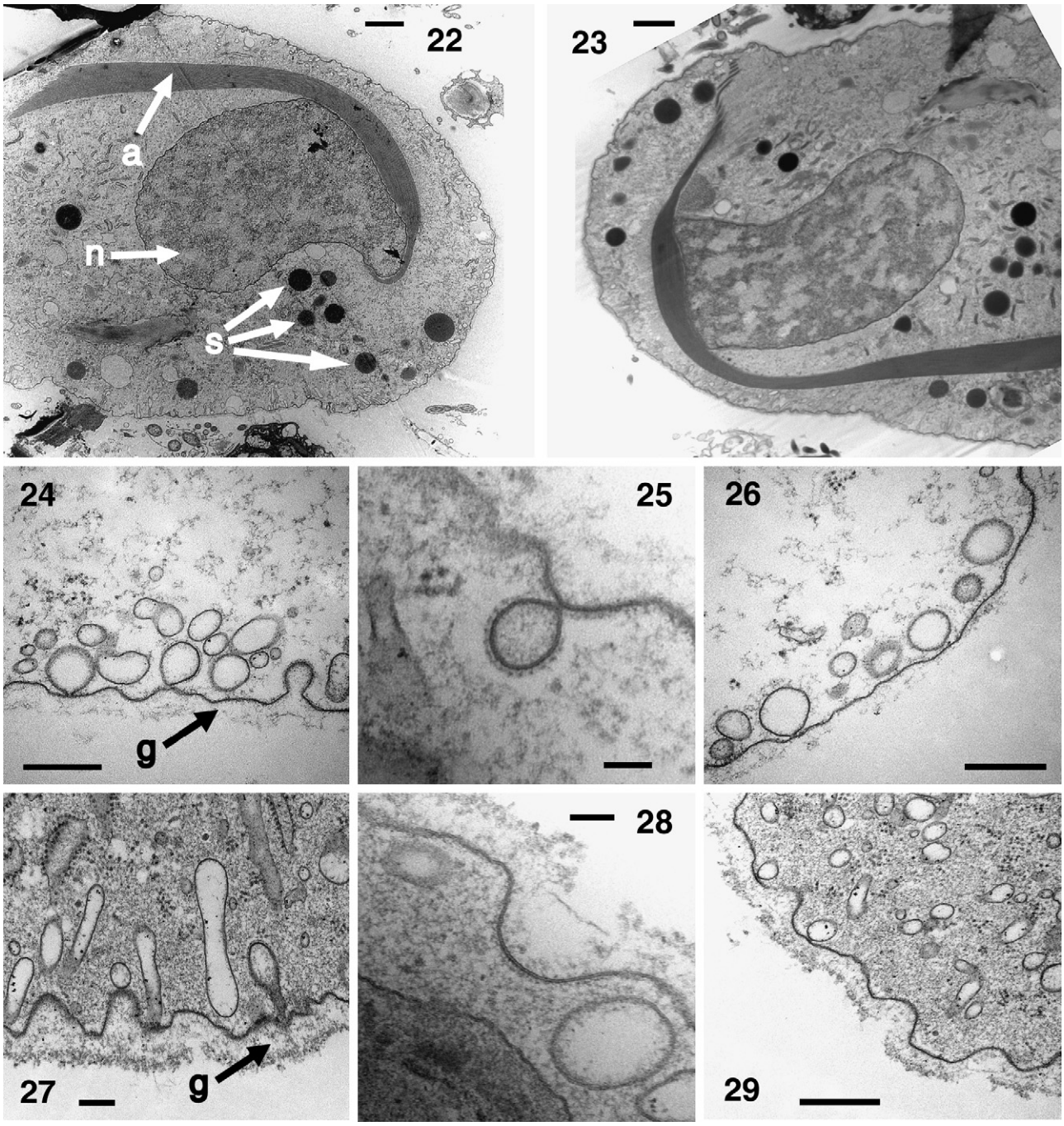
was not, and where present, it was much rarer than its two smaller congeners. It was never observed in SEM preparations, but was present in material examined with light microscopy (LM) and TEM (Fig. 22—32).

The most notable surface morphological feature of *S. minor* and *S. ambloaxostylus* observable in SEM is the presence of small concavities, with a raised circular or elliptical rim (generally 0.3—0.5 μm in diameter), and often bearing one or more small circular pores (roughly 50—100 nm) in the center (Figs 8—12). These concavities are usually distributed evenly over the cell's surface (Figs 1—5), but in *S. minor*, a minority of cells have irregular distributions in which some large areas lack them (Fig. 6). Some *S. ambloaxostylus* cells also exhibit somewhat uneven distributions, but to a lesser extent (Fig. 3). In some individuals of *S. minor*, small pores of about the same shape and size as those observed in the center of concavities appear in small numbers scattered over the cell surface, occurring alongside concavities (Fig. 13).

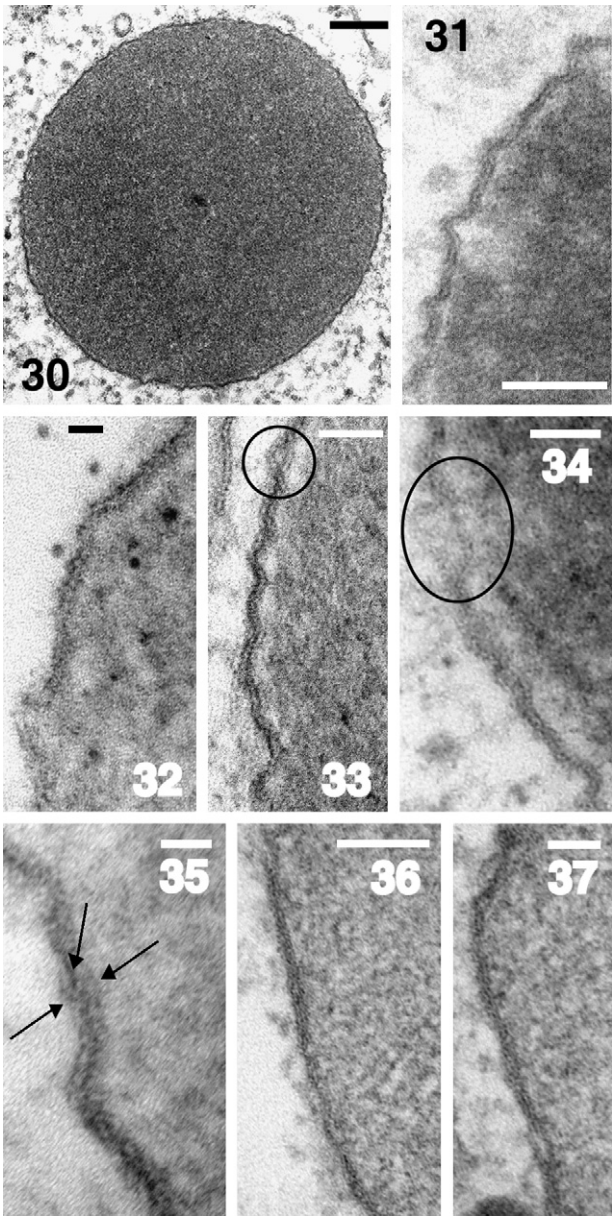
In TEM, *S. minor* and *S. ambloaxostylus* concavities appear as various shapes (i.e., as seen in section), from roughly circular (Fig. 14) to bowl-shaped (wide and shallow — Figs 15, 16), or somewhat square (Fig. 18). Hence, in many cases, the widest point of the concavity lies below its opening or aperture at the cell surface (Figs 15, 16). The pores observed at the base of the concavity by SEM can be seen in cross section (TEM) as small (about 50—100 nm in diameter) round pits (when continuous with the plasma membrane of the concavity) or vesicles (i.e., when just under the concavity) (Figs 14—17). Some pits are decorated with a regular coat of electron-dense material, suggestive of a clathrin coating (Fig. 16). Clathrin would have significant implications for the function of these pits, since clathrin-coated vesicles are indicative of endocytosis (Johannes and Lamaze 2002), and therefore reveal the direction of the vesicular transport. Vesicles of various shapes, often elongate tubules, not apparently associated with concavities are also common near the cell surface (Figs 14, 17), and decrease in frequency toward the center of the cell. The structure appearing as a raised circular or elliptical rim in SEM appears in sections (TEM) as the flared end of a column (Figs 14, 17), the column-like appearance resulting when two concavities are closely adjacent. Indeed, the concavities are so abundant on the surface of *S. ambloaxostylus* and *S. minor* that the outer edge of the cell in TEM has the appearance of an unzipped zipper (Figs 19—21). It is clear from TEM that the entire structure comprising the concavity is not extracellular but consists of cytoplasm bounded by plasma membrane (Figs 14—21). In some sections, concavities with no surface aperture are found. One interesting possibility is that concavities are opening and closing over time, but these images might also be sections through the edge of a concavity that is open elsewhere. In many TEM micrographs, a granular extracellular surface material (glycocalyx) roughly 50 nm thick coats the entire cell surface and is often seen spanning the aperture of the concavity (Figs 14, 15, 18, 19—21). This is not observed in SEM; the glcocalyx is difficult to preserve and visualize in SEM and is most likely removed during the critical point drying stage.

Saccinobaculus doroaxostylus was identified in TEM sections based on its large size, distinctive axostyle, and consistently anterior, pyriform nucleus (Cleveland et al. 1934; Heiss and Keeling 2006). In order not to attribute characters of *S. minor* and *S. ambloaxostylus* to *S. doroaxostylus*, we restricted our observations to sections where the large cell size was clearly evident and the nucleus and axostyle were visible (including close-up images in Figs 24—29). Interestingly, we found that *S. doroaxostylus* bears no concavities, but does have numerous coated pits and vesicles like those of *S. minor* and *S. ambloaxostylus* at or near the cell surface (Figs 24—29). As with *S. minor* and *S. ambloaxostylus*, there is a clear gradient in the frequency of vesicles — i.e., many vesicles are fused with, or near the plasma membrane, while fewer are seen toward the center of the cell (Figs 24, 26) — but the size and shape of the *S. doroaxostylus* vesicles are more variable. *S. doroaxostylus* also bears a glycocalyx comparable to that seen in *S. minor* and *S. ambloaxostylus,* except that it is frequently thicker, ranging up to 200 nm (Figs 24—29).

S. doroaxostylus also contains small (0.5—1.5 μm), roughly circular, membrane-bounded, electron dense bodies with no apparent internal structures (Figs 30—37). There are typically from a few to a dozen of these structures visible in each section (Figs 22, 23). It does not seem likely that these structures are phagocytosed bacteria because they always appear very nearly circular (i.e., there are no sections indicating they are rod-shaped bacteria or spirochetes), there is no indication that they are surrounded by a vesicle, and there is no sign of their being progressively degraded. It proved difficult to determine how many membranes surrounded these structures. In most



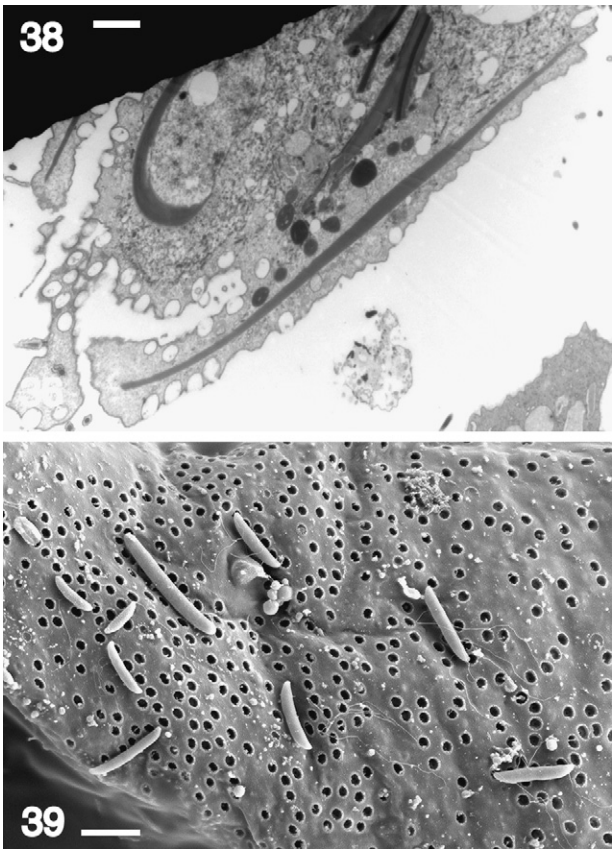
Figures 22—29. TEM micrographs of *Saccinobaculus doroaxostylus*. **22.** and **23.** Overviews of cells showing axostyle (a), nucleus (n), and unidentified, round, electron dense structures (s) (Scale bars = 2 μm). **24—26.** Sections near the plasma membrane showing the diversity in size and shape of numerous vesicles near the surface, and the overlying glycocalyx (g). Note molecules coating the outside of the vesicle in Fig. 25, which may correspond to clathrin (Scale bars: Figs 24, 26 = 500 nm; Fig. 25 = 100 nm). **27—29.** Sections near the plasma membrane showing vesicles and glycocalyx (g). (Scale bars: Fig. 27 = 200 nm; Fig. 28 = 100 nm; Fig. 29 = 500 nm).



Figures 30—37. TEM micrographs of unidentified, round, electron-dense organelles in *Saccinobaculus doroaxostylus*. **30.** Overview of a structure from the individual shown in Figure 22 (Scale bar = 200 nm). **31, 32.** Examples of organelles that appear to be bounded by a single membrane (Scale bars = 100 nm). **33—35.** Examples of organelles that appear to be bounded by a double membrane (Scale bars = 50 nm for Figs 33, 34; 20 nm for Fig. 35). Arrows indicate darkly-staining hydrophilic phosphate layers; circles indicate regions in which the two membranes appear to have separated. **36, 37.** Portions of a hydrogenosome of a parabasalid found in the same gut preparation as *Saccinobaculus doroaxostlyus* (Scale bars = 100 and 50 nm, respectively).

high-magnification images, the details of membrane structure were not well preserved in our fixes; however in some sections, a three-layer pattern of two dark-staining layers separated by a light layer was observed (Figs 31—32). This is indicative of a single membrane. In contrast, other sections showed a five-layer pattern where a very dark-staining layer is flanked on both sides by a light layer and a dark-staining layer (Figs 33—35). This pattern is indicative of two membranes that are closely appressed, and indeed, the same pattern was observed in hydrogenosomes from parabasalia in the same fixes (e.g., see Figs 36—37), which are known to be double membrane-bounded organelles derived from mitochondria. What's more, in some cases we also observed this five-layer structure separating into what appears to be two three-layer structures (circled in Figs 33—34), which is again suggestive of a closely-appressed double membrane separating into the two individual membranes. Altogether the identity of these organelles and the number of membranes that surround them are both still uncertain, but the presence of either a single membrane-bounded or double membrane-bounded organelle in this species is of interest. Oxymonads are one of the very few eukaryotic groups where there is still no evidence for either peroxisomes (single membrane-bounded organelles), or mitochondria (double membrane-bounded organelles). Based on the current data, it is our opinion that the balance of evidence, in particular the similarities between the membranes on these organelles and those of hydrogenosomes in the same preparation, suggests at least some of these organelles are candidates for relict mitochondria. Interestingly, an organelle with a very similar appearance has been described in *Trimastix*, the sister to oxymonads, and in some cases the membranes closely resemble the five-layer appearance described here (Figs 33—35) (Brugerolle and Patterson 1997; O'Kelly et al. 1999; Simpson et al. 2000). Either way, these organelles warrant further investigation as they could refine our understanding of the distribution of mitochondria or peroxisomes. We did observe one cell that bears both concavities and structures similar to these organelles (Fig. 38). This indicates that either *S. doroaxostylus* can form a few concavities on occasion, or that some *S. ambloaxostylus* cells have these possible mitochondrial derivative structures.

The surfaces of all three *Saccinobaculus* species examined here were generally devoid of bacteria (Figs 1—6). Exceptions include a few



Figures 38 and 39. Two exceptions. **38.** TEM micrograph of an unidentified member of *Saccinobaculus* bearing both concavities and the small, round, electron-dense orgenlles that we only identified in *S. doroaxostylus*. **39.** SEM micrograph of the surface of *S. ambloaxostylus* with several rod-shaped bacteria lying on top (Scale bars = 2 μm).

S. minor cells from one cockroach with a somewhat even but sparse distribution of rod-shaped bacteria on its surface (Fig. 7), and an occasional *S. ambloaxostylus* cell on which a dozen or so rod-shaped bacteria occur randomly over the surface (Fig. 39). Even these exceptions bear little similarity to the very dense and regular coating of rod-shaped surface bacteria (observable even with LM) characteristic of many other oxymonad species from termites, such as *Streblomastix strix* (Leander and Keeling 2004). Certain hypermastigote parabasalia from the same gut environment are regularly and densely covered with bacteria in our samples, (e.g., *Barbulanympha* and *Urinympha*), suggesting the lack of bacteria is not an artifact of specimen preparation. In those uncommon *Saccinobaculus* cells that do bear surface bacteria, there appears to be no relationship between the bacteria and the concavities, so the concavities do not seem to represent attachment sites for bacteria (note that bacteria in Figs 7 and 39 have no consistent orientation with respect to concavities).

We never observed any member of *Saccinobaculus* to have any attachment apparatus (i.e., a holdfast), or an anterior cell extension (a rostellum).

Discussion

Character Evolution

The addition of *Saccinobaculus* surface morphology data to equivalent data from representatives of the other four oxymonad families allows a more complete view of character evolution within oxymonads. Although phylogenetic relationships within the oxymonad clade are still not fully resolved, molecular data support a clade comprising Polymastigidae and Streblomastigidae to the exclusion of the other oxymonad families (Hampl et al. 2005; Heiss and Keeling 2006). Heiss and Keeling (2006) also recovered a weakly supported clade comprising the other three oxymonad families (Saccinobaculidae, Pyrsonymphidae, and Oxymonadidae), and the analysis of Hampl et al. (2005) was consistent with this (sequences from Saccinobaculidae were not available). Cleveland et al. (1934) also noted that members of these latter three families all have interphase nuclei that are very similar in structure. Altogether, data appear to be converging on this primary split in the oxymonad tree, so here we have used a consensus of these analyses as a framework for examining character evolution.

The tetraflagellate genus *Trimastix*, a free-living, heterotrophic inhabitant of anoxic waters, is likely the sister group of oxymonads based on molecular evidence (Dacks et al. 2001) as well as morphology (Simpson et al. 2002), and is included as the sister group to the oxymonad clade in our analysis. Morphological studies of *Trimastix* suggest that it lacks all of the structures discussed below — i.e., concavities, extracellular surface structures, a glycocalyx, bacterial surface attachments, a holdfast, and rostellum (Brugerolle and Konig 1997; O'Kelly et al. 1999; Simpson et al. 2000).

Concavities: The concavities we observed in *S. minor* and *S. ambloaxostylus* closely resemble those present in two of the other four oxymonad families — Pyrsonymphidae and Oxymonadidae. Maaß and Radek (2006) described "ringlike"

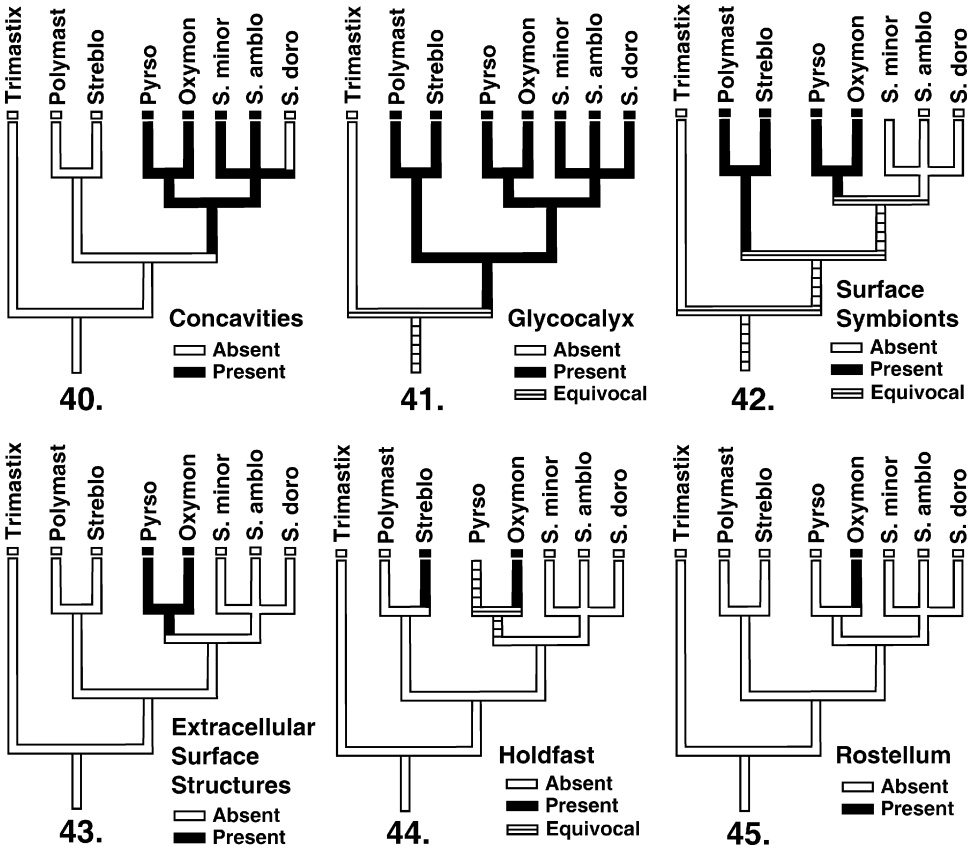
surface structures in an unidentified species of Pyrsonymphidae present in the gut of the termite *Neotermes cubanus*. Structures visible in their SEM micrograph of this species appear indistinguishable from those in *Saccinobaculus*, although the magnification is not high enough to discern whether the small pores (i.e., pits) we observed at the center of concavities in *Saccinobaculus* are present in this species. Rother et al. (1999) illustrated a similar surface structural complex (concavity, rim, central coated pit or vesicle, and glycocalyx) from two genera of the family Oxymonadidae (*Microrhopalodina* and *Oxymonas*). The sole member of family Streblomastigidae, *Streblomastix strix* from the termite *Zootermopsis angusticollis*, has been examined with both TEM and SEM (Hollande and Carruette-Valentin 1970; Leander and Keeling 2004), and shows no evidence of concavities anywhere on its surface. In the family Polymastigidae, members of the genus *Monocercomonoides* that have been investigated with TEM (Brugerolle and Joyon 1973; Radek 1994; Simpson et al. 2002) show no sign of concavities. The surface of *Polymastix melolonthae* does have a wave-like pattern in TEM (Brugerolle 1981), and while individual waves might possibly be construed as similar to a concavity in certain sections, these bear much closer resemblance to the ridges of *Streblomastix strix* (Hollande and Carruette-Valentin 1970; Leander and Keeling 2004), and in both species these function as sites for the attachment of symbiotic bacteria. Maaß and Radek (2006) provided SEM micrographs of a small tetraflagellate they classified as a member of family Polymastigidae from the termite *Neotermes cubanus*. This flagellate shows numerous holes in the surface, but unlike concavities in *Saccinobaculus,* some of these appear larger than most we observed (approx. 600 nm as opposed to 500 nm) and irregular in shape. They may represent artifacts seen in cells whose plasma membranes have been damaged in preparation for examination with SEM. Altogether, we believe the classification of this organism to the polymastigidae is not without doubt, and that additional examination with SEM and confirmation of the phylogeny of putative polymastigids will be necessary to clarify the issue.

We therefore propose the presence of concavities on the cell surface is a synapomorphy of the hypothetical clade comprising the families Saccinobaculidae, Pyrsonymphidae, and Oxymonadidae, with a secondary loss in *Saccinobaculus doroaxostylus* (Fig. 40). A small minority of *S. minor* cells have large areas lacking concavities (Fig. 6), and we cannot exclude the possibility that concavities could be altogether absent in certain life cycle stages. Because *Trimastix* lacks concavities, we conclude the ancestor of oxymonads also lacked them and, by extension, their absence in Streblomastigidae and Polymastigidae is ancestral. The distribution of this character is consistent with the weakly supported clade (based on molecular evidence) comprising Saccinobaculidae, Pyrsonymphidae, and Oxymonadidae recovered by Heiss and Keeling (2006).

Glycocalyx: A notably thickened glycocalyx, similar to that present in all three *Saccinobaculus* species, is also present in members of family Oxymonadidae — in *Microrhopalodina multinucleata* and *Oxymonas* sp. (Rother et al. 1999), and in *Microrhopalodina inflata* (Lavette 1975). A thick glycocalyx is likewise present in *Pyrsonympha vertens* (Pyrsonymphidae), but here it appears to include more highly modified structural components, which are described as umbrella-like (Lavette 1980). These appear to be the same structures as those described as scales by Smith and Arnott (1973) (see below), but Bloodgood (1974) referred to a glycocalyx as a separate structure from such “tiny projections”, hypothesizing that the latter may serve to anchor the former. The presence of a somewhat less thickened glycocalyx has also been noted in families Streblomastigidae (Leander and Keeling 2004) and Polymastigidae (Brugerolle 1981).

The wide distribution of the thickened glycocalyx in oxymonads suggests it is ancestral to the family. Its absence in *Trimastix* may reflect a loss in *Trimastix*, or a single origin in the common ancestor of oxymonads, perhaps as an adaptation to symbiosis in insect guts, thus representing a synapomorphy of that clade. Either reconstruction invokes one change of state (Fig. 41).

Surface Symbionts: The presence of bacterial surface symbionts is common among anaerobic flagellates in general (Fenchel et al. 1995; Finlay and Fenchel 1991), including oxymonads: bacteria covering some, or nearly all of the cell surface have been noted in Pyrsonymphidae (Rother et al. 1999; Smith et al. 1975), Oxymonadidae (Rother et al. 1999), Streblomastigidae (Hollande and Carruette-Valentin 1970; Leander and Keeling 2004), and Polymastigidae (Brugerolle 1981). It is somewhat unusual that members of *Saccinobaculus* — at least *S. ambloaxostylus* and *S. doroaxostylus* do not appear to form associations with bacterial surface symbionts. The lack of bacterial surface symbionts in these two species is confirmed here by light, scanning



Figures 40—45. Reconstructions of the evolution of six morphological characters in oxymonads. **40.** Concavities. **41.** Glycocalyx. **42.** Surface symbionts. **43.** Extracellular surface structures (ESS). **44.** Holdfast. **45.** Rostellum. Abbreviations: Polymast. = Polymastigidae; Oxymon. = Oxymonadidae; S. minor = *Saccinobaculus minor*; S. amblo = *Saccinobaculus ambloaxostylus*; S. doro = *Saccinobaculus doroaxostylus*. The topology used is from Heiss and Keeling (2006). Absence of a box under the taxon name indicates an uncertain coding for that character.

(*S. ambloaxostylus* only), and transmission electron microscopy (see Figs 1—3; 22—29 and Results). In *S. minor*, bacterial associations were only rarely observed (e.g., Fig. 7).

The harboring of epibiotic bacteria may have originated in the common ancestor of oxymonads with a loss in *Saccinobaculus*, or it may have originated independently in the Polymastigidae+Streblomastigidae clade and the Pyrsonymphidae+Oxymonadidae clade; either scenario invokes two changes of state (Fig. 42). This is complicated by *S. minor* — although most do not display any epibiotic bacteria, a few do, so we decided to code the taxon as uncertain. Choosing between the alternative reconstructions for these characters (or arguing in favor of a different hypothesis) will require further sampling of oxymonads for morphological data as well as improved resolution of relationships within the clade and evidence for the sister group of *Trimastix*+oxymonads. In addition, it would be of interest to determine whether the bacteria on the surface of Polymastigidae, Streblomastigidae, Pyrsonymphidae, and Oxymonadidae are closely related — possibly indicating an early association with an ancestral oxymonad host.

Extracellular Surface Structures (ESS): In addition to the glycocalyx, members of Pyrsonymphidae and Oxymonadidae have additional extracellular surface structures (ESS). Rother et al. (1999) described ESS covering the cell surfaces of *Microrhopalodina* and *Oxymonas* (Oxymonadidae), and proposed that they function as attachment sites for rod-shaped bacteria and spirochetes. The ESS are mushroom-shaped structures, each of which is associated with a concavity; the hollow, cylindrical stalk-like upright of the ESS contacts the plasma membrane inside

the concavity and the upper expanded surface functions as the attachment site for bacteria. Smith and Arnott (1973) noted two distinct types of ESS termed scales in *Pyrsonympha vertens* (Pyrsonymphidae). Both types comprise a hollow, elongate cylinder that attaches directly to the plasma membrane and supports a specialized distal portion, which may be divided into three or four branches (dendroid scale), or flared and trumpet-like (salpingoid scale). Thus, in their overall structure, they are comparable to the ESS reported by Rother et al. (1999) in Oxymonadidae, but differ in that they are smaller, narrow, elongate in shape, and do not appear to be associated with concavities or ectobiotic bacteria. Smith et al. (1975) found ESS in a species of *Dinenympha* (Pyrsonymphidae). These are described as screwlike, with the distal headlike portion forming an attachment site for spirochetes, and the shank inserted on the plasma membrane; however, in the absence of TEM micrographs, it is impossible to say whether these insert into a concavity. Extracellular surface structures are absent in investigated species of Streblomastigidae (Hollande and Carruette-Valentin 1970; Leander and Keeling 2004) and Polymastigidae (Brugerolle 1981; Brugerolle and Joyon 1973).

Extracellular surface structures appear to be a synapomorphy for the families Pyrsonymphidae and Oxymonadidae (Fig. 43), as they are absent in other oxymonads and in the common ancestor of oxymonads. This is consistent with the weakly — supported clade comprising these two families recovered by Heiss and Keeling (2006) as well as Hampl et al. (2005).

Holdfast or Rostellum: Some oxymonads have developed anterior structures specialized for attachment to the inner gut wall of their insect host. All such attached taxa have a microfibrillar holdfast, which contacts the gut wall, while Oxymonadidae have developed an additional anterior cell extension called a rostellum (connecting the holdfast and the bulk of the cell), which is largely microtubular (Brugerolle and Lee 2000). *Saccinobaculus* lacks both a holdfast and a rostellum, but the former structure is known in families Pyrsonymphidae (in *Pyrsonympha* but not in *Dinenympha*), Oxymonadidae, and Streblomastigidae, whereas a rostellum appears only in family Oxymonadidae (see Brugerolle and Lee 2000 and references therein).

The holdfast appears to have originated independently at least twice — in Streblomastigidae and in the common ancestor of Oxymonadidae and Pyrsonymphidae, with a loss in the prysronymphid genus *Dinenympha* (Fig. 44). It is also possible that it originated independently three times — in Streblomastigidae, Oxymonadidae, and the pyrsonymphid genus *Pyrsonympha*. Either scenario invokes three changes of state and reconstructs the common ancestor of oxymonads as lacking this character. The rostellum is known only from Oxymonadidae, thus representing an autapomorphy of that clade (Fig. 45).

Functional Considerations

The surface concavities of *S. minor* and *S. ambloaxostylus* invite speculation about their possible function, especially because the function of apparently homologous structures has been a source of speculation in other oxymonad families. It seems clear that concavities are closely associated with pinocytosis via formation of coated vesicles, not only in *Saccinobaculus*, but also in members of Oxymonadidae and Pyrsonymphidae (Rother et al. 1999). TEM micrographs of *Saccinobaculus* frequently show a close association with the glycocalyx, which is often seen arching over the external opening of the concavity (e.g., Figs 14—21). Because in some organisms, the glycocalyx is known to selectively accumulate certain solutes (food particles) from the surrounding medium via receptor molecules embedded within it (Hausmann et al. 2003), it is an interesting but untested possibility that the concavities in *Saccinobaculus* may serve as a reservoir for molecules absorbed by the glycocalyx, which will later be taken inside the cell via pinocytosis, thus forming a two-step concentration mechanism. Although, under LM, we observed at least some members of all three species of *Saccinobaculus* to have wood fragments within their cytoplasms (not shown), the abundance and apparently active nature of the vesicles suggest the cells are nevertheless dependent on the uptake of solutes to supplement their nutrition. Cleveland (1925) suggested that some oxymonads within insect gut environments do not contribute to lignocellulose digestion, but rather, exist by absorbing nutrients present as a result of the cellulose degrading activities of other microbial members of the community. In members of Pyrsonymphidae and Oxymonadidae, the process is likely somewhat different given that an ESS sits inside the concavity, where it has been proposed as an attachment site for epibiotic bacteria (Rother et al. 1999). The lack of bacterial ectobionts on *Saccinobaculus* suggests this is not a universal role for

the concavities; hence, other possibilities should be explored.

The general lack of ectobiotic bacteria in *Saccinobaculus* may be explained at least in part by the high levels of surface movement and (compared to other oxymonads at least) unparalleled plasticity of shape (see Cleveland et al. 1934; Heiss and Keeling 2006). This contrasts greatly with other members of the oxymonad clade such as *Streblomastix*, *Pyrsonympha*, and *Oxymonas*, which are covered with epibiotic bacteria, but remain attached to their host's inner gut wall by a holdfast, and generally undergo only minimal changes of shape (see Brugerolle and Lee 2000; Leander and Keeling 2004; Rother et al. 1999). One can easily imagine that in *Saccinobaculus* it may be difficult for bacteria to remain attached to such an active surface that undergoes such drastic changes in form over short time periods. In the *Cryptocercus* gut environment, a covering of epibiotic bacteria combined with a highly sedentary existence (i.e., attachment to the inner gut wall) may not confer the same benefits as in the termite gut; indeed this combination of traits is not seen in any protist member of this community.

Methods

Hindgut contents from individuals of the wood feeding cockroach *Cryptocercus punctulatus* were sampled, prepared, examined, and photographed with SEM as described previously (Carpenter and Keeling 2007). From each individual of *C. punctulatus* sampled, live *Saccinobaculus* cells were also observed with light microscopy (LM) using a Zeiss Axioplan 2 compound microscope with Plan Apochromat objective lenses and differential interference contrast (DIC) illumination.

For TEM sample preparation, microwave fixation was used as a rapid and effective way to achieve excellent ultrastructural preservation (Login and Dvorak 1994). *Cryptocercus* gut contents were suspended in 1 ml of Trager's medium (Trager 1957) plus 2.5% glutaraldehyde and microwaved, using a PELCO BioWaveTM Laboratory Microwave System under vacuum, twice for 2 min. Cells were washed four times with H_2O, post-fixed with 1% OsO_4 and microwaved as before, washed three times, then post-fixed with 2% uranyl acetate and microwaved as before. After two H_2O washes, cells were centrifuged into 1% low gelling temperature agarose (50 °C), cooled and excised as a small agarose block. An ethanol dehydration series of 50%, 70%, 95%, 100% with 40 s, Power Level 2 microwaving, was followed by 2X 100% exchanges and 40 s, Power Level 3 microwaving, all without vacuum. Epon:Spurr's (1:1) resin was infiltrated 25%, 50%, 75% and 100% three times each with microwaving under vacuum twice for 3 min with 3 min between. Resin was polymerized overnight at 60 °C. Thin sections (60 nm) were cut with a Leica Ultracut E Ultramicrotome, placed on formvar-coated grids and stained with 1% uranyl acetate and lead citrate. Grids were examined and photographed in a Hitachi S7600 transmission electron microscope at 80 kV.

Character evolution was studied with Mac Clade 4.07 software (Maddison and Maddison 2005). Characters from this study were mapped onto the oxymonad phylogeny inferred from SSU rRNA by Heiss and Keeling (2006). Character coding decisions were based on our own observations of *Saccinobaculus* along with data obtained from the literature (See Discussion) on other oxymonad taxa. The three species of *Saccinobaculus* were left as a polytomy because there is not sufficient evidence to support any hypothesis of relationships within this genus.

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