Ultrastructural Studies on *Selenomonas ruminantium* from the Sheep Rumen

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SUMMARY

The sheep rumen micro-organism *Selenomonas ruminantium* was studied in preparations of rumen contents by light- and electron-microscopic techniques. The fascicle of entwined flagella, which is often found curled-up close to the cell body, arises from a specialized organelle derived from the cell cytoplasm and cell membrane. This organelle has presumably developed from the 'polar organelle' specialization of the cell membrane already described for *Spirillum*, but in *Selenomonas* the organelle is situated directly behind the flagella instead of beside them. For this reason we use the distinguishing term 'flagellar organelle'.

Other structural peculiarities include flagellar fibres with unusually large diameter (20 nm) and 11-fold radial symmetry, an electron-dense layer adhering to the cell membrane in the region of the polar organelle, and an abundance of finger-like cytoplasmic processes projecting into an amorphous matrix beneath the layers of the cell wall.

The taxonomic status of *S. ruminantium* is briefly considered in the light of these results.

INTRODUCTION

The genus *Selenomonas* at present comprises three species: *S. palpitans*, *S. sputigena*, and *S. ruminantium*. The seventh edition of *Bergey's Manual of Determinative Bacteriology* (1957) has incorporated the genus into the family *Spirillaceae*. *S. ruminantium* is described as a large (up to 11 by 3 μm), rigid, motile crescent with a flagellar fascicle up to 10 μm long (visible under the light microscope). The fascicle arises from the central region of the concave side of the organism. The organism is Gram-negative, iodophilic, and a strict anaerobe. It often forms a major constituent of the flora of the rumen liquor of sheep and goats. The history of its nomenclature has been fully treated by the Editorial Board of the Judicial Commission on Bacterial Nomenclature (1955).

The taxonomic position of *Selenomonas* has not been satisfactorily elucidated; it has been classified as a bacterium by some workers and as a protozoan by others. It was discovered by Certes (1889), and recorded in detail by Woodcock & Lapage (1913), who described it as *Selenomastix ruminantium* in the belief that it was probably a protozoan. They also erroneously suggested that another common large rumen micro-organism (presently known as Quin's oval) represented part of the life cycle of *Selenomastix*. Wenyon (1926) favoured the name *Selenomonas* as proposed by von Prowazek (1913), and placed the organism in the protozoan family *Monadidae* (Kent). Lessel & Breed (1954) drew attention to the unusual type of flagellation in *Selenomonas* and also to the fact that the organisms occasionally appeared to move independently of any movement of flagella; in this respect they noted

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a resemblance to spirochaetes. Lessel & Breed (1954) also described a highly refractive granule in *Selenomonas palpitans* which stained with nuclear dyes and divided when the cell divided.

The granule of *Selenomonas palpitans* was also investigated by Jeynes (1955) who described it as a blepharoplast on account of its constant association with the base of the flagellum. Jeynes (1955, 1956) considered *Selenomonas* to be a protozoan.

Macdonald, Madlener & Socransky (1959) using dark-field microscopy and electron microscopy of shadowed specimens concluded that *Selenomonas sputigena* and *S. ruminantium* should be classed as bacteria, as they appeared similar to *Spirillum*; however, *Selenomonas palpitans* was considered to be a protozoan. Thin-sectioning studies were not part of this work.

Shimizu *et al.* (1968) studied shadowed and negatively stained ghost cells of *Selenomonas sputigena* with the electron microscope, but could not support the conclusion of Macdonald *et al.* (1959) that it had the characteristics of the genus *Spirillum*. Shimizu *et al.* (1968) showed that the apparently peritrichous flagellation found by Macdonald *et al.* (1959) was an artifact. In negatively stained organisms in favourable orientations the flagella were localized in the middle two-thirds of the concave side of the organism as described for *Selenomonas*.

From this condensed historical sketch it is evident that the taxonomic affinities of *Selenomonas* have proved confusing. We have studied one of the classical selenomonads, *S. ruminantium*, by electron microscopy of bacteria sedimented from crude, or partially purified, sheep rumen liquor. The fine structure of the organism will be presented, and an attempt made to clarify its taxonomic position. The helical twisting of flagella in the fascicle of *S. ruminantium* has already been described by Chalcroft (1971).

**METHODS**

Rumen liquor taken 2 h after feeding from sheep with a rumen cannula, and enriched with respect to the large bacteria (mainly *Selenomonas ruminantium* and Quin's oval organism), was prepared at the Biochemistry Department, Lincoln College, Christchurch, New Zealand, by the method of Wicken & Howard (1967). The material was fixed in 6 % (v/v) glutaraldehyde in 0.1 M-phosphate buffer, pH 7.2 (Millonig, 1961). The fixed preparation was refrigerated while being transported by air to Auckland, where the remainder of the study was carried out. There the phosphate buffer was replaced by several changes of veronal acetate buffer, before fixing the bacterial suspensions with osmium tetroxide and uranyl acetate (Ryter & Kellenberger, 1958).

Unfixed, crude sheep rumen contents were obtained from Dr D. E. Wright, Ruakura Animal Research Centre, Hamilton, New Zealand. This material was either fixed using the original osmium tetroxide and uranyl acetate Ryter–Kellenberger method alone, or combined with the glutaraldehyde prefixation as above.

Fixed or unfixed, enriched rumen liquor was negatively stained with a 1 % aqueous solution of ammonium molybdate.

For freeze-fracturing, fixed, enriched rumen liquor was mixed with glycerol to give a final glycerol content of 25 % (v/v), the specimen placed in small brass cups, then quick frozen and fractured by the method of Bullivant & Ames (1966).
RESULTS

Light microscopy of *S. ruminantium* in rumen fluid. The organism we studied corresponded closely with organism 4 in the photomicroscopic atlas of sheep rumen micro-organisms of Moir & Masson (1952). The organism was crescent-shaped with a flagellar fascicle arising from the centre of the concave side. The fascicle sometimes appeared as a straight or slightly curved appendage barely thick enough to be clearly recognized with the phase-contrast microscope. The fascicle was often curled up into a compact structure close to the organism and presented the appearance of a knob, circle, or spiral under the light microscope (Fig. 1). Living Selenomonas cells were observed with either extended or curled-up fascicles, therefore this feature appears to be no indication of either viability or death. Furthermore, the curled-up fascicles are not necessarily caused by centrifugation procedures as suggested earlier by Chalcroft (1971).

If an unfixed preparation of Selenomonas was allowed to stand for an hour or so before phase-contrast examination, a plate-like structure could often be resolved close to the organism surface on the concave side beneath the fascicle.

The fine structure of thin-sectioned *Selenomonas ruminantium*. The outermost layer of the organism (the capsule) was a dark, fibrous coat, about 55 nm thick (Fig. 2). The structure of the cell wall was typical of that of Gram-negative bacteria (de Petris, 1967), with five layers, three of which stained heavily. The innermost layer (cytoplasmic membrane) did not occupy the normal position in close apposition to the cell wall. Instead, it was strongly convoluted and often some distance within the organism. A layer of lightly stained, amorphous material filled the intervening space. The average thickness of the layer was 200 nm. It was partly occupied by finger-like or vesicular protrusions of the cytoplasm, of average diameter 100 nm, bounded by unit membranes presumably continuous with the cell membrane (Fig. 3). These structures will be referred to as cytoplasmic processes.

Ill-defined, ribosome-like material occurred in patches which were generally close to the cytoplasmic membrane. Lightly stained nucleoplasmic regions occupied the remaining central part of the organism. Individual flagella were long (up to 10 μm) and thick (20 nm diam.), arising in a group of between 20 and 50, and twined together helically a short distance from the organism to form the cable-like tuft visible as a ‘flagellum’ with the light microscope. The tuft was often observed twisted into a ring close to the concave side of the organism. When individual flagella were sectioned transversely to their axes, they displayed considerable substructure (Fig. 4).

In all cases where flagellar substructure was visible, there was a central core of 12 nm diam., surrounded by eleven equally spaced subunits which extended to the outer circumference of the flagellum. The photographic enhancement technique of Markham, Frey & Hills (1963) was applied to some of these cross-sectioned flagella, and confirmed the 11-fold radial symmetry.

At the proximal end of the flagella tuft, individual flagella were separated from each other and inserted singly in a hexagonal pattern into a restricted area at the centre of the concave side of the organism (Fig. 5). Each flagellum terminated in a bullet-shaped base (50 nm by 70 nm), which extended through the thickness of the cell wall and also through the bounding membrane of the flagellar organelle described below (Fig. 6).

The flagellar organelle appeared as a flattened sac situated between the protoplasmic cylinder and the cell wall in the middle of the concave side of the organism. It was bounded by a unit membrane. Its membrane might be continuous with the cytoplasmic membrane and the organelle may have cytoplasmic continuity with the remainder of the organism.
Fig. 1. *Selenomonas ruminantium* from an unfixed sheep rumen preparation photographed with oil immersion, phase-contrast optics. Note typically curved shape, coarsely granular cytoplasm, and the dark plate at the base of the curled-up flagellar fascicle in the centre of the concavity.

Fig. 2. Thin section of *Selenomonas ruminantium*. Note crescentic morphology, peripheral cytoplasmic processes (CP) and the specialized flagellar organelle (FO) with its associated darkly stained plate (P). The curled-up flagellar fascicle is sectioned at various orientations to its main axis.

Our electron micrographs of longitudinally sectioned cells sometimes suggested that the organelle is a tongue of differentiated cytoplasm lying along the long axis of the bacterium (Fig. 2); however, the cytoplasmic continuity across the ‘neck’ region was not entirely convincing, owing to the presence of electron-dense material and membraneous material, which made interpretation of the course of the bounding membrane ambiguous in this region. The organelle occupied about one-third of the length of the organism on the concave side.
Fig. 3. Cell periphery at high magnification. Note wall layers typical for Gram-negative bacteria, also the extensive layer of amorphous material (A) penetrated by membrane-bounded cytoplasmic processes (CP).

Fig. 4. *Selenomonas ruminantium* flagellum seen in cross-section (arrowed). Note the central solid core surrounded by 11 peripheral subunits.

Fig. 5. A thin section of *Selenomonas ruminantium* oriented obliquely to the hexagonal array of flagellar bases. The section has passed through the outer, darkly stained, cell capsule and has also grazed the plate-like structure associated with the flagellar organelle. Note hexagonally close-packed array of subunit particles comprising the plate, P.
Fig. 6. A longitudinal section of *Selenomonas ruminantium* showing bullet-shaped flagellar bases inserted into the flagellar organelle.

Fig. 7. A flagellar organelle at high magnification showing details of the plate. The plate is composed of fine, short fibrils attached to the cytoplasmic surface of the unit membrane which surrounds the organelle. Another densely stained layer applied closely along the exterior surface of the membrane opposite the fibrils is arrowed.
and was always associated with the flagellar bases (Fig. 6). Its cytoplasm appeared rather different in texture from that of the bacterium proper. The external flat side of the organelle bore the flagellar bases, while the internal flat side bore on its cytoplasmic aspect a plate-like structure composed of a two-dimensional array of short, heavily stained fibrils. This plate was approximately 17 nm thick and up to 1.5 μm long. In apposition to the fibrillar plate, but on the other side of the membrane, was a thin heavily-stained layer about 3 nm thick. A clear space 2 nm thick separated it from the membrane (Fig. 7). In near-grazing sections of the plate (Fig. 5), the structural units (i.e. the short fibrils) could be seen as a darkly stained hexagonal array. The contents of the vesicle were more granular
Fig. 9. A negatively stained *Selenomonas ruminantium* cell in division. Two flagellar fascicles with hexagonal close-packed arrays of bases can be seen.

Fig. 10. Negatively stained *Selenomonas ruminantium* flagella. In each flagellum, four to five longitudinal rows of subunits may be seen.
Fig. 11. Frozen-fractured *Selenomonas ruminantium*. Note particle-studded fracture faces of the cytoplasmic processes, CP. Smooth fracture faces in the cytoplasm are also visible.

Fig. 12. Frozen-fractured *Selenomonas ruminantium* showing a fracture face of the flagellar organelle membrane (FO). On this particle-studded face may be seen the outlines of fractured flagellar bases. Some distance out from the flagellar bases are the curved outlines of the flagellar fascicles.
than the remainder of the cytoplasm and there were often unstained regions near the flagellar bases.

Organisms in division were occasionally observed. As in other Gram-negative bacteria (Steed & Murray, 1966) the cell wall seemed to be synthesized within the invaginations of the cytoplasmic membrane, to form a centripetally growing division septum. The flagellar organelle divided with the rest of the organism. We have no evidence that either it or the flagella disappeared at any stage of division (Fig. 8).

The fine structure of negatively stained Selenomonas ruminantium. Large crescents were seen which had long flagellar tufts wound in coils close to the cell bodies. The crescents were occasionally found oriented in such a way that the insertion bases of the flagella could be clearly recognized as hexagonal arrays on the central region of the cytoplasmic membrane (Fig. 9).

When the negatively stained coiled flagella were examined at higher magnifications, evidence of longitudinal striations along the fibres was often visible (Fig. 10). At least four rows could be discerned. Flagella detached from the cells often showed enlarged, hook-like ends where they were previously attached.

The fine structure of freeze-fractured Selenomonas ruminantium. The morphology of Selenomonas demonstrated by freeze-fracturing did not differ substantially from that shown by thin sectioning. It often appeared crescent-shaped, and could always be distinguished from other bacteria in the suspension because it possessed many small cytoplasmic processes near the periphery. The fracture faces of the membranes of these processes bore a covering of closely packed particles about 7 nm in diameter (Fig. 11). Other smooth fracture faces seen in the organisms probably represent lipid globules.

In some fractured cells, the profile of the flagellar organelle (already described for thin-sectioned bacteria) could be seen. Cross-fractured flagellar tufts typical for Selenomonas were often seen in the frozen extracellular ice, close to the concave sides of the crescent-shaped organisms.

Occasionally the fracture face of the exterior flagellar organelle membrane with its associated flagellar bases was revealed. The flagellar bases appeared as shallow craters with raised rims consisting of about 15 particles (Fig. 12).

DISCUSSION

We do not consider this large rumen selenomonad to be the same organism as the small crescent-shaped strains isolated from bovine rumen contents and also named Selenomonas ruminantium by Bryant (1956). Although Purdom (1963) and Hobson, Mann & Smith (1962) interpreted their experiments as indicating identity between the large and small crescents, Prins (1971) has recently grown pure cultures of both large and small crescent forms from sheep and has shown that the two forms are quite distinct. A micrograph of a negatively stained organism of Prins’ S. ruminantium strain ss appears identical to the large selenomonad described in this paper.

The bacterium which we have identified from morphology alone as Selenomonas ruminantium displays a number of unusual features.

The origin of the cytoplasmic processes in the space between the cell wall and the bulk of the cytoplasm is not known. Vesicular structures have been noted in microcysts of Myxococcus xanthus following addition of glycerol to the growth medium (Bacon & Eiserling, 1968). Those seen in Selenomonas ruminantium might have occurred during pre-fixation with glutaraldehyde. However, their appearance in frozen-fractured cells eliminates the possi-
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...bility that they are artifacts resulting from the subsequent stages of embedding and thin-sectioning. We regard the flagellar organelle as a real structure because it is unlikely that such a well-organized body could result from invagination of the cytoplasmic membrane during our preparative procedures.

The flagellar organelle, which almost certainly corresponds to the 'refractive granule' of Lessel & Breed (1954), or to the 'blepharoplast' of Jeynes (1955), is far more complex than the organelle associated with flagella described in other bacterial groups. It is, however, possible to imagine such a structure evolving by the invagination and elaboration of a 'polar membrane' region such as that described for *Spirillum serpens* by Murray & Birch-Anderson (1963).

The flagellar organelle is not similar in morphology to the eukaryote blepharoplast. The corresponding granule of *Selenomonas palpitans* was claimed to be a blepharoplast by Jeynes (1955) on the basis of its behaviour during cell division. The flagellar organelle does have a positional similarity to the blepharoplast in that it is found close to the cell motility organelles, and its reproduction is controlled so that it divides in synchrony with that of the whole cell.

The flagella are unusually thick and complex compared with those of other bacteria. The *Selenomonas ruminantium* flagellum has a diameter of 20 nm and shows 11-fold radial symmetry, readily seen in thin sections. The unsheathed flagella of most other bacteria have diameters of the order of 12 nm and after negative staining show radial symmetry ranging from 5- to 10-fold (Kerridge, Horne & Glaubert, 1962; Lowy & Hanson, 1965). For comparison, microtubules of eukaryote cells have diameters of about 27 nm and exhibit 13-fold radial symmetry (Ledbetter & Porter, 1964; Gall, 1966). It is interesting that the axial fibres of some spirochaetes have diameters (15 to 20 nm) approaching that of *S. ruminantium* flagella, although they only show vague substructure in thin sections (Bharier, Eiserling & Rittenberg, 1971; Listgarten & Socransky, 1964).

From our studies on the ultrastructure of *Selenomonas ruminantium* we conclude that this organism is not a protozoan. We believe that the present classification of *Selenomonas* by Breed et al. (1957) as a genus of the bacterial order *Pseudomonadales* is satisfactory, but several of the unique morphological features described above suggest that *S. ruminantium* should not be grouped with *Spirillum* in the *Spirochaetaceae* but should be transferred to a separate family. A similar situation may exist for *Selenonomas palpitans*.

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