

Isolation of *Herpetosiphon giganteus* and Ultrastructure Analysis by Electron Microscopy

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ABSTRACT

Herpetosiphon giganteus is a filamentous gliding bacterium. Gliding motility is the movement of the cells over surfaces without the aid of flagella. The mechanism responsible for bacterial gliding motility has not been known and there are only a few data on *Herpetosiphon giganteus*. The aim of this study was to observe the ultrastructure and negative staining of isolated strains of *Herpetosiphon giganteus* to find any organelles of locomotion. First, 35 strains of gliding bacteria were isolated from soil, freshwater, mud and activated sewage sludge. Then, 8 strains very closely related to *Herpetosiphon giganteus* were used for further examination. For extracellular slime and fibril observation, photoelectron micrographs were taken from different patterns on the cell surface of strains that were negatively stained. Thin sections with and without lysozyme treatment were prepared and examined by transmission electron microscopy. When the filaments were negatively stained, fibrils were detected in young cultures. There were two different kinds of fibrils in this study. The extracellular slime of these organisms was clearly visible. Examination with the electron microscopy revealed neither flagella nor an axial filament of any kind. There was no evidence for external organelles of locomotion. The results indicated that ring like structure localized at the cell surface connected with fibrils is responsible for gliding movement. The secretion of slime is necessary for adhesion of the cell to a solid surface and for ease of movement. *Iran. Biomed. J. 8 (1): 19-23, 2004*

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INTRODUCTION

A new genus, *Herpetosiphon*, isolated from soil was established by Holt and Lewin [1], to include organisms that produced unbranched, filamentous, Gram-negative, non-flagellated bacteria with a slow gliding motility on a solid surfaces. Many microorganisms including myxobacteria, cyanobacteria and flexibacteria move by gliding [2].

There are three major mechanisms of bacterial surface translocation: gliding, twitching and swarming. Although the mechanism of *Myxococcus xanthus* (soil bacteria) gliding is not known, studies have indicated that *M. xanthus*-gliding motility is regulated by two genetically separate systems: A (adventurous) and S (social) [3]. Numerous experiments and reports have demonstrated the absolute requirement of polar type IV pili for *M. xanthus* S motility. The removal of pili either by genetic mutations or by mechanical shearing leads to S motility defects [4, 5]. The *dif* locus is required for the biogenesis of extracellular matrix fibril that is crucial for S motility. Through various assays, *dif* mutants are defective in fibril biogenesis [6].

Videomicroscopic studies on gliding movement, as well as ultrastructural observation of two myxobacteria suggest that the A-system motor may consist of multiple single motor elements that are arrayed along the entire cell body. Each motor element is proposed to be localized to the periplasmic space and to be anchored to the peptidoglycan layer. The force to glide, which may be generated here, is coupled to adhesion sites that move freely in the outer membrane [7].

Genetic studies indicated that the *dif* genes are linked to the *M. xanthus dsp* region, a locus known to be crucial for *M. xanthus* fibril biogenesis and S gliding motility [6]. The type IV pilus of *M. xanthus* functions as a motility apparatus. Pili were required for *M. xanthus* cells to adhere to solid surfaces and to generate cellular movement using S motility [8]. Type IV pili retract, generate substantial force and directly mediate cell movement [9, 10].

Gliding motility is the movement of the cells over surfaces without the aid of flagella. The mechanism responsible for bacterial gliding motility has not been known. There are only a few data on *Herpetosiphon giganteus*. Negative staining is particularly valuable for the examination of bacterial flagella. In shadowing method, a thin layer of heavy metal deposit on the surface of specimen. When an electron beam is passed through the coated preparation in the electron microscope and positive print is made from the negative image, a three dimensional effect is achieved. The aim of this study was to observe the ultrastructure and negative staining of 8 isolated strains of *Herpetosiphon giganteus* to find any external organelles of locomotion.

MATERIALS AND METHODS

Bacterial strains. *Herpetosiphon giganteus*, strain *Hpa-2* was obtained from Deutsche Sammlung Von Mikroorganismen (German Collection of Microorganisms, D-3400. Gottengen, Grisebachstr, 8, under Code number DSM 589). This bacterium used as standard bacteria for comparing the 35 strains of gliding bacteria isolated from different sources. In this study, only 8 strains very closely related to *Herpetosiphon giganteus Hpa-2* were used for further examination. These 8 isolated strains of *Herpetosiphon giganteus* are: 1 strain (S1) from soil, 1 strain (S2) from freshwater, 2 strains (S3, S4) from mud, 3 strains (S5-S7) from activated sewage sludge and 1 strain (S8) from horse dung.

The electron micrographs were taken with an AEI Corinth 500, transmission electron microscope used at an accelerating voltage of 80kv loaded with film Agfa F710p. Thin sections were made with an L.K.B. model ultratome (R) 1118800, with a knife maker L.K.B. model 7800B.

Herpetosiphon isolation was essentially similar to the method used by Soriano [11]. Briefly, A small amount of soil was streaked on an bacto agar plate contained CaCl_2 and nystatin and then the plate was incubated at 25°C. Among the mixed growth of organisms, which

developed on the surface of this medium, some colonies were distinguishable by their edges and rough fibrous appearance. On diluted agar media, gliding bacteria tend to spread out from particles of debris and to form diffuse, veil like colonies. By taking advantage of this ability of the organisms to spread over a solid substrate, it was possible to establish pure cultures by removing filaments from the edge of such colonies. The method used for ultramicroscopy was according to Hoppert and Holzenburg[12].

Negative staining. For extracellular slime and fibril observation, cells grown on CYB (30 g/l Casitone, 1.0 g/l yeast extract, 1.0 g/l CaCl_2 , 15.0 g/l Bacto agar) plates were suspended in distilled water. The suspension was placed on carbon coated electron microscopy grids and the excess distilled water was removed with filter paper. Grids were negatively stained with uranyl acetate for a few minutes and examined using the electron microscopy. The diameters of fimbriae were measured directly on enlarged electron photo-micrographs of the negatively stained and chemical extraction of cell preparation, using a metric ruler. The width and length of the filament were measured on both electron photomicrograph and enlarged photomicrographs of the Gram-stained preparation using a metric ruler. Photoelectron micrographs were taken from different patterns on the cell surface of strains that were negatively stained.

Shadow preparation. In order to achieve a three- dimensional effect, shadow preparation were made. The filaments were suspended in distilled water and placed on the Formvar coated grids. The grids were set an angle of 15°C , 12 cm from the source, which was comprised of 2.5 cm of 20% palladium/80% platinum wire, wrapped round a tungsten filament. When a vacuum of 10^{-4} torr or better had been reached in the chamber, a current was passed through the tungsten filament and increased slowly until the palladium/platinum wire melted and evaporated. The grids were observed using a transmission electron microscopy.

Fixation, embedding and sectioning. Samples were taken from 7-day-old cultures on CYB agar. For this sectioning, the samples were placed in 2% agar and glutaraldehyde solution (2.5% in phosphate buffer at pH 7.0). The samples were fixed for one hour and then rinsed 3 times over 15 minutes, i.e. the buffer was changed every 5 minutes. The preparations were fixed with osmium tetroxide in phosphate buffer for one hour, followed by 3 further rinses.

The samples were dehydrated through graded acetone, 30, 50, 70, 90 and 100%, 5 minutes in each grade, with three changes of 100% acetone. Then, the specimens were placed in 1:1 spur resin, acetone mixture. After 24 hours, the specimens were placed in resin for one hour. The resin was changed and left overnight and then was embedded. After dehydration, the sample was infiltrated by an embedding resin. For embedding, the infiltrated agar cubes sank to the bottom of the vial. Two volumes of resin added to the sample and incubated for 1.5 h, changed with fresh resin and incubated for 2 h. Single cubes transferred into small beam capsule and then polymerized at 60°C for 24 hours. Thin sections (100 nm) were cut and placed on electron microscopy grids. Thin sections were stained with saturated aqueous uranyl acetate solution for 20 minutes, followed by Reynolds' lead citrate, for 4 minutes. After each treatment, the sections were rinsed with distilled water and excess fluid was drawn off with filter paper. The grids were air-dried and observed using a transmission electron microscopy.

Lysozyme treatment. Cells, taken from 7-day-old CYB plates, were suspended in BELS solution, (potassium-sodium phosphate buffer, pH 7.0 containing sucrose, lysozyme and EDTA). Magnesium sulphate was added to give a final concentration of 0.05 M, and after a few minutes, an additional amount of sucrose was added and incubated for 75 minutes. The lysozyme-treated cells were centrifuged and fixed as above for electron microscopy.

Chemical extraction of cells. The method of Pate and Chang [13] was used for this experiment. Cells were harvested from 7-day-old CYB plates resuspended in buffer 1,

containing (Tris, EDTA, DDT [dithiothreitol]). Cells suspensions were homogenized on ice in a glass tissue homogenizer, 50 strokes by hand and the cells were removed by centrifugation for 15 minutes. The pellet was discarded and the supernatant was centrifuged for 3 hours. The supernatant was dialyzed overnight against buffer (5 mM N-Tris [hydroxymethyl] methyl-2-amino-methane sulphonic [TES] pH 7.0; 0.2 mM ATP; 0.2 mM DDT; 0.1 M KCl, 5 mM MgCl₂), which was designed to keep actin in its fibrillar form. The dialysate was centrifuged for 3 hours to obtain a supernatant and pellet. Pellet was negatively stained by placing a droplet of material on an electron microscopy grid (carbon coated), rinsing the grid with uranyl acetate, drawing off excess uranyl acetate solution with filter paper and air-dried. The grids immediately examined using the electron microscopy.

RESULTS

Negative staining. When the filaments were negatively stained, fibrils were observed in young one-week-old cultures. They were thin and usually present as twisted bundles. The fibrils ran randomly in all directions along the cell surface and formed a network. The extra cellular slime of these organisms was clearly visible in electron micrographs of negatively stained whole cells of young cultures. Where slime was visible, the fibrils occurred in patches (Fig. 1).

Fig. 1. Electron micrograph of negatively stained preparation of *Herpetosiphon giganteus*. S, Slime; F, Fibril; R, Ring; Bar represents 0.1 μm .

Cells shadowed with carbon and platinum are shown in Figure 2. There was no evidence for external organelles of locomotion. No surface structures such as flagella were seen but fibrils could be observed. There appears to be a very thin slime layer surrounding the cells. Cells in these preparations were quite opaque to the electron beam, so that internal structures were not visible.

Ultrastructure. Thin sections were prepared and examined by transmission electron microscopy. The septate filaments were surrounded by an envelope of the usual Gram negative appearance outside the membrane and separated from it by a small gap. There was an inner single dense layer, which continued with constant thickness into septa. Outside the dense layer, a rather wide layer, although relatively diffused, could be seen.

Fig. 2. Electron micrograph of shadowed preparation of *Herpetosiphon giganteus*. F, Fibrils; S, Slimes; Bar represents 0.1 μm .

The diffuse layer did not enter the septa properly, although it followed closely the surface of the cells at the sites of attachment of septa. The surface of the diffuse layer (i.e. the surface of the cell), was usually rather irregular and having a hairy appearance. The septum appeared to be composed of the cell membrane plus electron dense material which was continuous with an inner layer of the cell wall and was probably composed of peptidoglycan, since this material was not present after lysozyme treatment. The outer cell wall layer did not take part in the formation of the septum; thus cross wall formation was not complete in long filaments.

No layers were in any of the sections examined, which ran smoothly across the annular constriction as would be expected with a sheath.

Cells contain extended system of intracytoplasmic membranes. Some of them were rather delicate and obviously connected with septa; they may be regarded as true mesosome. Mesosomes were seen in various areas of the cell and frequently were observed attached to septa in different stages of completion. Mesosomes were frequently seen associated with and sometimes attached to both developing and complete septa.

The average width of 46 filaments was 0.35 to 0.7 μm on enlarged electron micrographs of strains tested. The length of the cells was variable, usually 1.5 to 5 μm . No evidence of the production of spores or other resting stages was found. Examination with the electron microscopy revealed neither flagella nor an axial filament of any kind.

Chemical extraction. When cells of all strains were fractionated, ring-like structures were found in fraction P3. Ring-like structures in one strain (S1) was slightly larger (0.4 μm against 0.3 μm in diameter), and numerous fine fibrils could be discerned. These fibrils were not present in the extracts of the other strains.

The diameter of fibrils was 7.57 to 15.15 nm in the chemical extraction of the cells compared with 5.20 to 10.14 nm in the negative staining techniques on enlarged electron micrographs of isolated bacteria.

DISCUSSION

Our results showed that the fine structure of the isolated bacteria (*Herpetosiphon giganteus* S1-S2) indicated that the outer membrane of the cell envelope could not be resolved as a separate structure, probably because it is fused with underlying dense (peptidoglycan) layer. There was an additional wall layer outside this membrane, membrane-peptidoglycan complex, but not a sheath in the classical sense, as postulated by the original definition of the genus, was lacking. This confirms the results obtained by Reichenbach and Golecki [14].

In this investigation, using phase contrast microscopy, it was impossible to detect either septa or cross walls in the filaments without prior staining. The difficulty in visualizing these structures may reside in the narrow width of the filaments, (less than 1 μm). Staining of the filaments clearly showed the filaments containing several septa. Electron microscopy studies of thin sections also revealed septa.

Mesosomes were observed frequently in this investigation, especially in lysozyme treated cells. However, it was observed that the filaments of the organisms examined were of indefinite length, unbranched, definitely no sheaths and exhibiting bending and gliding movements on solid surfaces. Filamentous means multicellure in this case.

In this study, the electron microscopy of negatively stained preparations indicated that the all strains (S1-S8) tested produced abundant amount of slime with fibrils (5.20-10.41 nm). However, in the chemical extraction of cells, all strains examined possessed ring like structures but only in one strain (S1), fibrils were associated with ring like structures. The fibril diameter of strain S1 in chemical extraction was 7.57-15.15 nm. The explanation for this phenomenon is that the fibril structures of different strains (S1-S8) are different, so, there was at least two different kinds of fibrils in this studies (negatively stained and chemical extraction). The diameter of fimbriae in some gliding bacteria has reported 6.4-10.8 nm by Mac Rae *et al.* [15].

It seems reasonable to assume that different gliding bacteria might have different fibril structures and rotary assemblies arranged in different pattern on their surfaces, just as different pattern exist for flagella insertion in bacteria. Such differences might explain great variation in abilities of different gliding bacteria to move. There may also be variations in kinds of rotary assemblies among the gliding bacteria. For example, the goblet shaped assemblies covering surfaces of cells *Flexibacter polymorphus* [16], may be rotary assemblies with extensions on them that may assist motility in certain kinds of environment.

It is obvious that a great deal of experimental data on gliding have been gathered since 1960. None of them is sufficient in itself to provide a comprehensive explanation of the gliding phenomenon. It seems likely that ring like structure, localized at the cell surface and connected with fibrils is responsible for gliding movement. The secretion of slime is necessary for adhesion of the cell to a solid surface and for ease of movement.

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