Flagella, Gas Vacuoles and Cell-wall Structure in *Halobacterium halobium*; an Electron Microscope Study

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SUMMARY: A droplet of a suspension of *Halobacterium halobium* in a concentrated brine when left to dry on a collodion-covered grid yields a useful preparation for electron microscopy. Two strains, one red and one pink, were examined. Both strains have polar tufts of flagella. The cell wall contains a single layer of hexagonally arranged globular particles. *Halobacterium halobium* has these characteristics in common with *Spirillum* spp., but unlike the true *Spirillum* spp. it is rod-shaped.

Electron microscopical examination showed that the gas vacuoles observed by Petter (1931, 1932) to be a feature of pink strains of *H. halobium* actually were smaller but much more numerous than the optical microscope revealed. Stereopictures show that these vacuoles did not collapse upon desiccation of the bacteria.

Red discoloration of salted fish can be caused by at least three distinct species of bacteria. In most cases a rod-shaped bacterium has been isolated from the substrate. *Bacillus halobius ruber* is the name Klebahn (1919) gave to a red rod-shaped halophilic bacterium. Petter (1931) changed the name to *Bacterium halobium* because it does not form spores. According to her, *Pseudomonas salinaria* Harrison et Kennedy is the same species. More recently, Elazari-Volcani (1940) attached the generic name *Halobacterium* to it, *H. halobium* being the type species.

In 1954, Mr de Melker, working in the Laboratory for Microbiology at Delft, obtained some strains of this species. Originally the colonies of all the strains were transparent and red. Some of the strains, however, on being subcultured for some time on proteose peptone NaCl (23%, w/v) agar, began to make opaque pink colonies. This was what Petter had described in her preliminary paper (1931) and in her thesis (1932). In other respects also Mr de Melker's strains did not seem to differ from those which Petter had examined. Petter made a thorough investigation of the characteristics of several strains of the bacteria of salted fish. She found that the red and the pink strains of the rod-shaped species had in common a mixture of two closely related carotenoid pigments. The macroscopically different appearance of cultures of the two strains was paralleled by a different appearance under the microscope: the pink organisms contained a variable number of 'bodies' with a low refractive index while the red organisms did not. The results of several experiments led Petter to the conclusion that these 'bodies' in the pink bacteria consisted of, or contained, gas. (Compare the phase-contrast micrographs, Pl. 1, figs. 1, 2). The main argument was, and still is, that these 'bodies' can be made to disappear by subjecting a suspension of pink bacteria to a pressure of a few atmospheres. The result is immediately visible: the suspension turns red.

Microscopical examination shows that no 'bodies' are left, so the opacity as well as the different colour is clearly due to the presence of these 'bodies'. The latter Petter named 'gas vacuoles' as she considered them comparable to the gas vacuoles which Klebahn had described in Cyanophyceae. Though Petter collected other evidence that the 'bodies' in her bacteria were indeed gas vacuoles, some later authors have disagreed with this interpretation. The fact that different authors have used different strains may partly explain the contrary views. Spruit & Pijper (1952) examined a strain having a morphological form which showed many twisted cells. A comparison of the image observed in their microscope with the micrographs on p. 45 of Petter's thesis made them apply to the appearance of Petter's organisms an interpretation of their own, namely, that the represented bacteria were twisted organisms and not organisms containing gas vacuoles. Spruit & Pijper's interpretation might have differed less from Petter's, had they studied the latter author's strain directly, focusing up and down on the organisms as she did. According to a later paragraph in Spruit & Pijper's article, not all of the images observed by Petter should be explained by assuming all the organisms to be twisted; some might contain highly light-scattering particles. To call these bodies 'products of degeneration' does not, however, discharge the authors of the duty of explaining the unusual physical properties which led Petter to infer that these 'bodies' were gas vacuoles.

METHODS

Because of the presence of the gas vacuoles, the specific weight of bacteria of the pink strain is less than that of a concentrated brine (e.g. 23 %, w/v, NaCl). When a suspension of these bacteria in 23 % NaCl solution is centrifuged, a pink layer forms on top of the supernatant fluid. However, only organisms originally near the surface gather in this layer. The other organisms are subjected to so much pressure that this causes the gas vacuoles to collapse. Therefore centrifugation also yields a red pellet, and is not a suitable method to use when pink bacteria are to be prepared for electron microscopical examination.

For another reason the usual procedure of washing the organisms with distilled water before desiccation on the collodion membrane cannot serve in this case. The organisms turn into a slimy mass as soon as the medium is diluted with water. If this bacterium could be adapted to a medium containing only a little NaCl, such organisms might perhaps survive suspension in distilled water. It has been found, however, that a medium with less than c. 12 % (w/v) NaCl does not support growth.

Good results were obtained with organisms taken directly from the pink layer floating on top of an undisturbed liquid medium. Small drops of this layer were diluted with a 27 % (w/v) NaCl solution; a droplet of the suspension was placed on top of a collodion-covered grid and partly sucked off with filter-paper. It now proved an advantage that these bacteria were adapted to a concentrated brine: during desiccation the cells found themselves in natural surroundings up to the last moment.

After desiccation the specimens were shadowed with platinum. Electron micrographs were taken with a modified Philips E.M. 100 instrument, which has a short focal-length objective lens designed by Le Poole. Although most of the organisms were to a greater or lesser extent obscured by small NaCl crystals, a small percentage of organisms escaped being hidden in this way and electron micrographs were made from these. Stereopictures were taken by tilting the object by *c.* 7 degrees to the right and as much to the left, alternately.

For the present investigation one red and one pink strain were selected from the strains of Mr de Melker.

RESULTS

Flagella

A high percentage of the organisms showed motility, though the movements were restricted to axial rotation and oscillation of the poles. Some micrographs (see Pl. 1, figs. 3, 4) show bacteria with polar tufts of flagella. This is in agreement with the findings of Harrison & Kennedy (1922) and of Lochhead (1934) who succeeded in staining the flagella. Though other authors failed in an attempt to make these organelles visible, none but Spruit & Pijper (1952) have claimed that this apparently motile bacterium lacks flagella.

Gas vacuoles present in the pink strain

In electron micrographs of bacteria, e.g. *Mycobacterium tuberculosis* (Knaysi, Hillier & Fabricant, 1950; Brieger, Cosslett & Glauert, 1954; Scanga, 1954) and *Caulobacter vibrioides* (Bowers, Weaver, Grula & Edwards, 1954) ordinary vacuoles are known to show up as less dense areas. In the present investigation the same was found with the gas vacuoles. There were, however, some differences. Ordinary vacuoles become quite flat upon desiccation. The adjacent cytoplasm contains a lower percentage of water and therefore flattens less. Micrographs of shadowed specimens show that the less dense areas are simply the flattest areas of the bacterial body; the upper cell wall has sunk down to the bottom of what looks a shallow pit and which is the site of the collapsed vacuole.

The vacuoles of the pink bacteria, as shown in Pl. 4, fig. 1, apparently do not collapse; the site of a vacuole is hardly flatter than the adjacent cytoplasm.

Stereopictures (Pls. 2, 3) give a much better insight into the three-dimensional structure of the desiccated bacteria. On original prints the observer sees the upper cell wall as nearest, and the gas vacuoles in various planes, in some places one behind the other.

The original volume of the gas vacuoles will not necessarily have been decreased by desiccation. Their shape, on the other hand, may have altered. The cytoplasm has lost water, and therefore the whole of the bacterial body, gas vacuoles included, must have flattened. At the same time the cytoplasm around the gas vacuoles will have contracted also in the horizontal plane. Both causes will have co-operated in making the gas vacuoles lens-shaped instead of spherical, and therefore in making them look very large. Unfortunately this results in the micrographs falsely suggesting the organism to be

almost filled with gas vacuoles, and the latter to be separated from one another by little more than a membrane. This appearance may cause objections to be raised against the method used in preparing the specimens. One may doubt, however, whether any other method of dehydration would result in a truer representation of the bacterium.

The distances between many of the gas vacuoles must be rather small since the optical microscope appears unable to show all of them as separated. The electron microscope shows how large their number really is.

The lesser density with respect to the medium, as shown by the ability of the pink organisms to float on top of it, is but one consequence of the presence of gas vacuoles. Since the gas vacuoles fill part of the space within the cell wall, another consequence is that the ratio 'volume of the cytoplasm/surface area of the cell wall' is smaller than it would be if the gas vacuoles were lacking. This ratio is also determined by the shape of the organism. In ribbon-shaped organisms, for example, the ratio is smaller than in cylindrical ones. In this connexion it is interesting that Spruit & Pijper (1952), studying a red strain of *Halobacterium halobium* which lacks gas vacuoles, found 'that the majority of the individuals did not have the usual cylindrical form of bacteria, but were more or less flat, bent, or curled ribbons'. Also in the present investigation flat organisms were found in great numbers in cultures of the rod-shaped halophilic bacterium, especially with the red strain. Therefore the red strain of *H. halobium* has, in common with the pink, a smaller ratio 'volume of the cytoplasm/surface area of the cell wall' than would be found if the organisms were cylindrical and if they lacked vacuoles. In growing organisms the same must be true of the ratio 'newly formed cytoplasm/newly formed cell wall material'; it follows that both in vacuolated and in flat organisms the proportion of assimilated nutrients turned into cell-wall material is comparatively large. It is quite another question whether a large surface area with respect to the volume of the cytoplasm is profitable to the organism. There was found no indication that this was the case with *H. halobium*.

The cell wall

Some of the micrographs (Pls. 2, 4) of the pink organisms, as well as some of the red organisms, show a cell-wall structure very similar to that of *Spirillum* spp. (Houwink, 1953). Globular particles about 130 Å. wide are arranged in one layer in a hexagonal pattern. If the globular particles in the bacterial cell wall are macromolecules—and this does not seem too wild a speculation—this layer of the cell wall may be a two-dimensional crystal lattice. Since a crystal grows by apposition only, and not by intussusception, this cell wall will have definite growth zones, coincident with the borders of the crystalline areas. Such borders must occur at the poles of the cell, where the shape of the cell wall passes from the tube into the dome. Additional growth zones may exist in other parts of the cell wall, wherever a dislocation occurs in the regular pattern of the globules. A similar cell-wall structure has not been found in any *Pseudomonas* sp. thus far. This may be one reason to consider *Halobacterium*

halobium more closely related to the genus *Spirillum* than to the genus *Pseudomonas*.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Phase-contrast micrograph of *Halobacterium halobium*, red strain. Magnification, $\times 1500$.
- Fig. 2. Phase-contrast micrograph of *H. halobium*, pink strain. Magnification, $\times 1500$.
- Figs. 3, 4. Electron micrographs of *H. halobium*, pink strain. Tufts of flagella. Magnification, $\times c. 35,000$.

PLATE 2

Stereo electron micrograph of *H. halobium*, pink strain. Magnification, $\times c. 60,000$.

PLATE 3

Stereo electron micrograph of *H. halobium*, pink strain. Magnification, $\times c. 52,500$.

PLATE 4

- Fig. 1. Electron micrograph of *H. halobium*, pink strain. Magnification, $\times c. 60,000$.
- Fig. 2. Electron micrograph of *H. halobium*, red strain. Magnification, $\times c. 60,000$.
- A simple stereoscope may be made of two identical magnifying glasses with a diameter of *c.* 6 cm. and a focal length of *c.* 20 cm. The distance between the glasses should be adjustable to the observer's need.

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A. L. HOUWINK—GAS VACUOLES AND CELL WALL IN *HALOBACTERIUM*. PLATE 1

(Facing p. 150)

A. L. HOUWINK—GAS VACUOLES AND CELL WALL IN *HALOBACTERIUM*, PLATE 2