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Gliding Motility in *Aphanothece halophytica*: Analysis of Wall Proteins in *mot* Mutants

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The unicellular cyanobacterium *Aphanothece halophytica* (PCC 7418) is motile, and spontaneous nonmotile (*mot*) mutants accumulate when the organism is subcultured. Analysis of *mot* mutants suggests that a glycoprotein in the cell wall is involved in the motility mechanism. Proteins from the wall fraction of the wild type and five *mot* clones were analyzed by gradient sodium dodecyl sulfate-acrylamide gel electrophoresis. Four clones were similar to the wild type, and one clone, *mot-3*, was missing a high-molecular-weight protein (~200,000) and had at least one new polypeptide (160,000). The high-molecular-weight protein stained with periodic acid-Schiff reagent, suggesting that it was a glycoprotein. The absence of the protein in *mot-3* did not affect the mechanical strength of the wall, since both *mot-3* and wild-type cells were broken at the same rate by controlled cavitation. Several other cyanobacteria were also screened for the presence of glycoproteins. All motile strains have such proteins, although none had an apparent molecular weight as high as that in *Aphanothece* sp. Some motile strains, such as *Oscillatoria limnetica* and *Phormidium* sp., showed very large amounts of glycoprotein; whereas some nonmotile strains, such as *Synechococcus* sp. (UTEX 625) and *Microcystis* sp. (PCC 7820), showed no high-molecular-weight glycoproteins.

The mechanism of gliding motility in the gram-negative cyanobacteria has been studied principally in filamentous strains in which both the physiology and the energetics of the process have been characterized (1). There are no obvious appendages associated with such motility at the light microscope level, and there are no regular shape changes which occur when the filament is in motion. However, ultrastructural studies indicate that motility may involve a series of oriented fibrils located in the L-3 layer of the wall, and suggestions have been made that standing waves somehow generated in the wall layers may be directly involved in the generation of motion (8, 9). Much of what is known about the mechanism of motility in flagellated bacteria comes from an analysis of nonmotile (*mot*) mutants (5). However, there are no similar studies of the properties of *mot* mutants of the gliding cyanobacteria, and as yet, no specific proteins have been identified which are both necessary and sufficient for wild-type motility.

Aphanothece halophytica (PCC 7418, ATCC 29534) is a unicellular gas-vacuolated halophilic cyanobacterium which was originally isolated in 1972 from Solar Lake, located south of Elat, Israel (2). The organism is motile and forms spreading colonies on plates. During studies on the plating characteristics of *A. halophytica*, it was noted that spontaneous *mot* mutants could be isolated. An analysis of the characteristics of one such *mot* strain is reported here. It is shown that motility is associated with the presence of a high-molecular-weight glycoprotein which is located in the wall fraction. In addition, it is shown that proteins of this type are commonly associated with the character of gliding motility in the cyanobacteria.

MATERIALS AND METHODS

Culture conditions. *A. halophytica* was grown at 35°C in the ASN-III medium of Rippka et al. (13) modified to include 75 g of NaCl per liter, 6 g of $MgCl_2 \cdot 6H_2O$ per liter, and 1.5 g of KCl per liter. Standing liquid cultures were swirled twice daily and incubated under cool-white fluorescent lamps. *A. halophytica* was plated on Chu-11 medium (18) modified by the addition of the following (grams per liter): NaCl, 56.3; KCl, 1.34; $MgCl_2 \cdot 6H_2O$, 11.02; $MgSO_4 \cdot 7H_2O$, 13.84; and $CaCl_2$, 2.9. The medium was solidified with 0.5% (wt/vol) agar. Plates were sealed with Parafilm to prevent excessive dehydration and were incubated at 35°C in the presence of unidirectional cool-white fluorescent light. *Oscillatoria limnetica* (3) and a *Dactylococcopsis* species isolated from Solar Lake (from A. E. Walsby, Department of Botany, University of Bristol, Bristol, England) were also grown on the

modified ASN-III medium described above, whereas the several species of freshwater cyanobacteria used were grown on the BG-11 medium of Stanier et al. (18). Freshwater strains were obtained from either the University of Texas Culture Collection, the Paris Culture Collection, or the American Type Culture Collection or were gifts from the isolator.

Cell fractionation and wall isolation. Cells (5×10^8 to 1×10^9) were fractionated after breakage by blending in a Vortex mixer in the presence of glass beads. Cells were pelleted at 8,000 × *g* for 10 min and suspended in 1.25% (wt/vol) NaCl–10 mM Tris (pH 8.0) (TN). Cells were washed once with TN and finally taken up in 1.6 ml of TN in a 15-ml Corex tube (Corning Glass Works). After 6.8 g of 0.1-mm-diameter, acid-washed glass beads was added, the tube was blended at top speed in a Vortex mixer for four 1-min intervals. Each period of blending was separated by 1 min of cooling on ice. No intact cells were observed microscopically after this step. Beads were allowed to settle, and the liquid was transferred to a new tube. The beads were washed with four 1.5-ml portions of TN, and all washes were pooled with the initial recovered liquid. Cell walls were collected by centrifugation at 200 × *g* for 5 min and washed once with TN. Walls were finally suspended in distilled water, and samples were prepared for electron microscopy or frozen for later analysis of wall proteins.

Total cell proteins were prepared by resuspending a cell pellet in a small volume of distilled water and cavitating the sample with the microprobe on a Branson Soniter until intact cells were no longer observed microscopically. No attempt was made specifically to inhibit proteases. However, all extracts were kept at ice temperature during preparation, and when completed, samples were boiled in the presence of 1% sodium dodecyl sulfate (SDS) and 0.1% (vol/vol) β-mercaptoethanol and frozen until analysis.

Acrylamide gel electrophoresis. Discontinuous SDS-acrylamide slab gels were prepared by using the system of Laemmli (10) modified for the preparation of gradient gels (17). The stacking gel contained 5% (wt/vol) acrylamide, and the running gel was composed of a continuous gradient of 7 to 20% (wt/vol) acrylamide. The electrophoresis buffer (pH 8.3) contained 0.025 M Tris-hydrochloride, 0.192 M glycine, 0.001 M EDTA, and 1% (wt/vol) SDS. Samples to be electrophoresed were made 0.01 M Tris-hydrochloride (pH 6.8), 0.001 M EDTA, 2.0% (wt/vol) SDS, 4.6% (vol/vol) glycerol, 0.1% (vol/vol) β-mercaptoethanol, and 0.01% (wt/vol) bromphenol blue. Samples (25 to 100 μg of total protein or 5 μg each of molecular weight markers) were heated to 100°C for 3 min before loading on the gels. Samples were electrophoresed at 35 V (constant) until the sample dye marker reached the bottom of the gel. Gels were then removed from the apparatus and stained.

Total proteins were identified by staining with Coomassie blue R-250 as previously described (16). Periodic acid-Schiff reagent was used to stain gels for glycoproteins by using the procedure of Segrest and Jackson (15). Stained gels were photographed with a Polaroid MP-4 camera, using positive/negative type 665 film. Molecular weight standards included: β-galactosidase, 131,000; bovine serum albumin, 65,000; ovalbumin, 45,000; DNase I, 31,000; trypsinogen, 24,400; and cytochrome *c*, 12,500. In addition, total cell extract from *Halobacterium salinarium* strain 5 was used as a periodic acid-Schiff staining marker since this species contains a single high-molecular-weight glycoprotein (190,000) (12).

Measurements of relative wall strength. Relative wall strength of various *A. halophytica* isolates was characterized by determining their sensitivity to disruption by sonic oscillation. Cell suspensions (6 ml each) containing 1.5×10^6 to 2.0×10^6 cells per ml were cavitated with the microprobe tip of a Branson Sonifier (setting no. 1, 30 W) for various lengths of time. At intervals, 0.1-ml samples were removed and diluted 1:10 with fresh growth medium. Intact cells per milliliter remaining at each time interval were determined by counting cells in a Spiers-Levy counting chamber with a Zeiss microscope at ×256. Results are expressed as percent cells remaining intact after a given dose (minutes per milliliter of solution cavitated) of sonic oscillation. The geometry of the apparatus, including tube shape and probe depth, was kept constant between experiments, and the samples were kept on ice during treatment.

Miscellaneous assays. Isolated walls were prepared for electron microscopy by placing a drop of wall suspension on a carbon-coated collodion-covered grid and then washing the grid successively with horseheart cytochrome *c* (1 mg ml^{-1}), water, and 1% (wt/vol) uranyl acetate. The liquid remaining from the last wash was removed by blotting with filter paper. After drying, the grids were examined in a transmission electron microscope.

To test the sensitivity of wall fractions to enzymatic digestion, 100-μg protein samples of total cell extract were treated at 37°C overnight with either 2% (wt/wt) lysozyme, 2% (wt/wt) trypsin, or 2% (wt/wt) pronase. Lysozyme and pronase digestions were carried out in 0.05 M Tris-hydrochloride (pH 8.0), whereas trypsin digestions were done in 0.05 M Tris-hydrochloride–0.005 M $CaCl_2$ (pH 8.0). Protein was measured using bovine serum albumin as a standard.

The pressure-induced collapse curves for gas vesicles were determined by monitoring the pressure-induced changes in culture turbidity (19).

RESULTS

Wild-type cells of *A. halophytica* plated on modified Chu-11 medium containing 0.5% (wt/vol) agar and incubated in the presence of unidirectional light formed asymmetric spreading colonies indicative of both limited motility and phototactic movement toward the light source (Fig. 1a). This strain, when subcultured for several years, accumulated *mot* mutants which formed compact colonies when plated under the same conditions as the wild type (Fig. 1b). Subclones of these variants remained nonmotile, and spontaneous revertants back to the wild-type motility were not seen. The frequency of the spontaneous appearance of *mot* mutants was $<10^{-5}$ per division and was not precisely determined because of the difficulties associated with

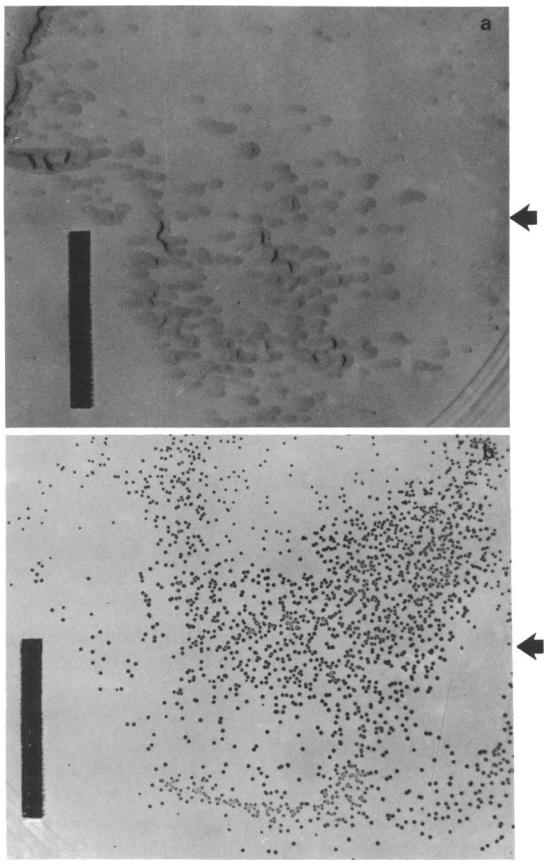

FIG. 1. *Photographs of A. halophytica colonies grown on 0.5% agar plates containing modified Chu-11 medium. The plates were incubated in the presence of unidirectional light, the source of which is indicated by the arrow. (a) Wild-type A. halophytica; (b) plated cells of the nonmotile mot-3 clone. Bar = 2 cm.*

characterizing strains with an unselectable marker.

The total proteins from five clones of spontaneous *mot* mutants were analyzed on 7 to 20% gradient acrylamide-SDS gels. It was observed that four of the clones had protein patterns identical to the wild type, whereas one clone was missing a high-molecular-weight (<200,000) protein-staining band, but had at least one new protein-staining band. The latter clone, designated *mot-3*, was analyzed in greater detail.

The altered protein species were localized in the wall fraction (Fig. 2). Overnight digestion with pronase or trypsin removed all Coomassie blue staining material, whereas overnight digestion with lysozyme had no effect on the patterns of proteins seen after electrophoresis (data not shown). The protein species which is missing in *mot-3* stained with periodic acid-Schiff reagent, whereas the additional protein species in *mot-3* did not (Fig. 3).

Wall fractions of the wild-type strain and *mot-3* were examined in the electron microscope (Fig. 4). The procedure used for wall isolation primarily produced three characteristic types of wall structures: half-cell envelopes (Fig. 4a and b), curled wall fragments (Fig. 4c), and sheets with curled edges (Fig. 4d). Wall structures of all three types could be found in preparations from both *mot* and *mot*$^+$ strains, although specific quantitative differences were not determined. There were no obvious differences in the surface features (textures) of the two types of wall preparations, and no external wall features such as fimbriae were observed.

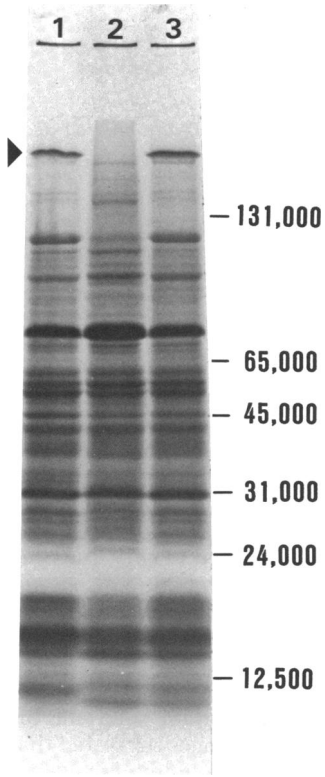

FIG. 2. *Gradient acrylamide-SDS gel electrophoretic separation of wall proteins from the wild type and mot mutants of A. halophytica. (1) Wild-type wall proteins; (2 and 3) proteins from mutant clones mot-3 and mot-2, respectively. The 7 to 20% gels with a 5% stacking layer were prepared and run as described in the text and were stained with Coomassie blue R-250. The positions of known molecular weight markers electrophoresed under the same conditions are given on the right-hand side. An arrowhead designates the position of the polypeptide which is missing in mot-3.*

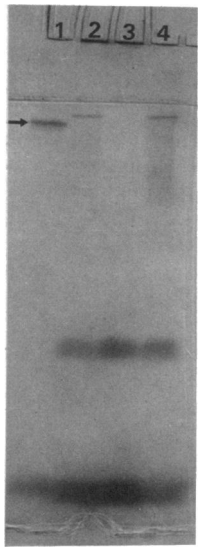

FIG. 3. *Gradient acrylamide-SDS gel electrophoretic separation of A. halophytica wall proteins stained with periodic acid-Schiff reagent. The 7 to 20% gels with a 5% stacking layer were prepared as described in the text. Lane 2 contains an extract of wild-type wall proteins, and lanes 3 and 4 contain extracts from mutant clones mot-3 and mot-2, respectively. As a standard, lane 1 contains a total cell extract from H. salinarium strain 5, which has been shown to contain a 190,000-dalton glycoprotein (12) (arrow).*

The mechanical strength of the cell wall in isolates of *A. halophytica* was tested by analyzing the resistance of intact cells to sonic oscillation. No significant differences were noted in the ability to disrupt intact cells of either the wild type or the *mot-3* mutant (Fig. 5).

All clones analyzed in this study are gas vacuolate. The *mot-3* clone produced greater numbers of gas vesicles than did the wild type since the cells in standing cultures of *mot-3* accumulated at the surface of the culture, whereas under the same conditions, cells of the wild type were uniformly suspended in solution. Pressure-induced turbidity changes suggested that the *mot-3* mutant had, at a minimum, 25% more gas vesicles than did the wild type. The pressure-induced collapse curves for the wild type and for the *mot-3 A. halophytica* were identical (Fig. 6).

A series of both motile (gliding) and nonmotile cyanobacteria was screened for the presence of glycoproteins. Total cell extracts were dissolved in the SDS-containing sample buffer and electrophoresed on 7 to 20% gradient acrylamide-SDS gels. The gels were stained with periodic acid-Schiff reagent (Fig. 7). Glycolipid material which electrophoresed as diffuse spreading bands was found in all samples tested. However, sharp high-molecular-weight staining bands were found in all of the motile cyanobacteria tested, including *O. limnetica* (3) and a *Phormidium* species. Although neither had a glycoprotein with an apparent molecular weight as large as that in *A. halophytica*, the glycoprotein component was one of the more abundant proteins present in the total cell extract. In the *Phormidum* species examined, Coomassie blue staining (not shown) indicated that the glycoprotein constituted 0.5 to 1.0% of the total protein. The nongliding species *Anabaena variabilis* (ATCC 29143), *Dactylococcopsis* sp. (from A. E. Walsby), *Microcystis* sp. (PCC 7806), and *Synechococcus* sp. (UTEX 625) did not show high-molecular-weight glycoproteins, whereas the nonmotile *Gloebacter violaceus* (PCC 7421) showed a faintly staining band with an electrophoretic mobility similar to that identified in *O. limnetica*.

DISCUSSION

A. halophytica is one of a limited number of unicellular cyanobacteria which are capable of some form of gliding motility (7, 18). The organism is phototactic, since spreading colonies form asymmetrically in the direction of the incident illumination (Fig. 1a). Movement over sterile agar toward a unidirectional light source was initially used to produce axenic clones in this strain (Y. Cohen, Ph.D. thesis, Hebrew University, Jerusalem, Israel, 1975). The limited motility of this microorganism relative to many filamentous strains (1) is a technical advantage because distinct colonies are formed which are morphologically distinguishable from those produced by nonmotile variants (Fig. 1). Thus, it is possible to determine quantitatively the frequency of motility variants, and it should be possible to identify phototaxic mutants, since they would form symmetrical spreading colonies in the presence of unidirectional light. No spontaneous variants of the latter type have been seen in the approximately 10^5 colonies examined in the studies reported here.

Although Castenholtz (1) described the appearance of nonmotile mutants of *Oscillatoria terebriformis*, the work reported here represents the first biochemical analysis of such a class of mutants. The *mot-3* mutant of *A. halophytica* lacked a single high-molecular-weight wall protein normally found in the wild-type strain. The missing component was a glycoprotein, because it was digested by proteases, it was stained with periodic acid-Schiff reagent, and its deletion in *mot-3* involved the loss of both the Coomassie blue staining (protein) and the periodic acid-Schiff staining band. Because glycoprotein mo-

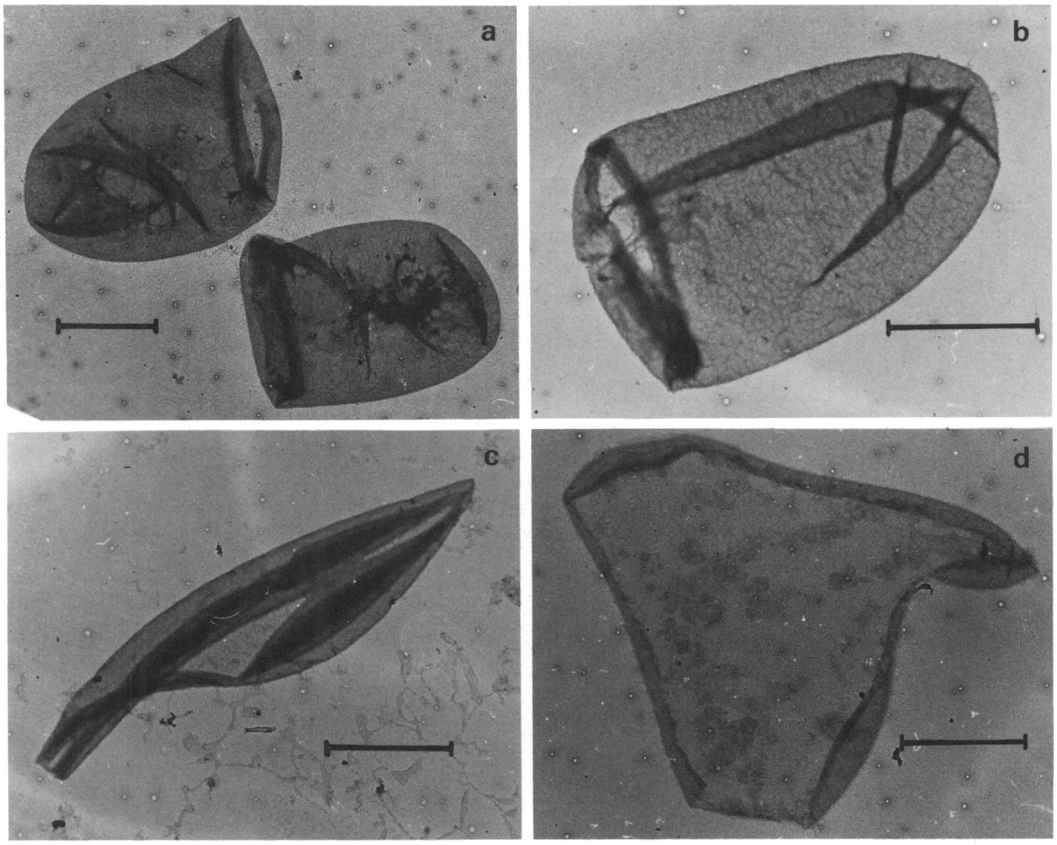

FIG. 4. *Electron micrographs of A. halophytica wall fragment forms isolated from the wild-type motile strain. Wall fragments were isolated and prepared for electron microscopy as described in the text. Bar = 2 μm.*

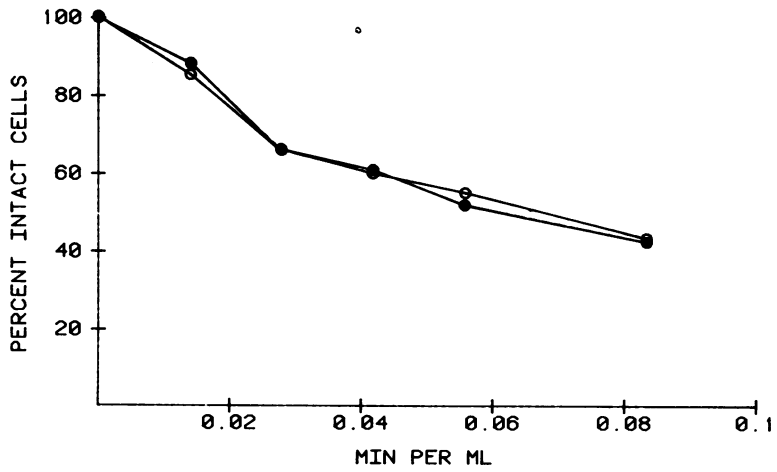

FIG. 5. *Mechanical strength of A. halophytica walls as measured by the sensitivity of intact cells to controlled sonic oscillation. Cell suspensions of the wild type (●) and mot-3 (○) were treated with increasing doses (minutes per milliliter) of sonic oscillation, and the percent intact cells remaining after each dose were determined as described in the text.*

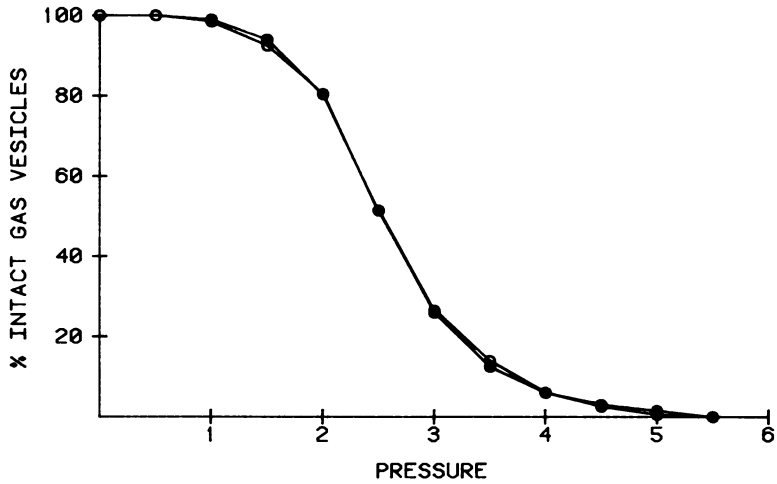

FIG. 6. *Pressure-collapse curves of gas vesicles in the wild type (●) and mot-3 (○) mutant of A. halophytica suspended in culture medium. The percentage of gas vesicles collapsed was determined by measuring the change in culture turbidity which resulted from treating the cultures with increasing pressure as described by Walsby (19). Pressure is given in bars.*

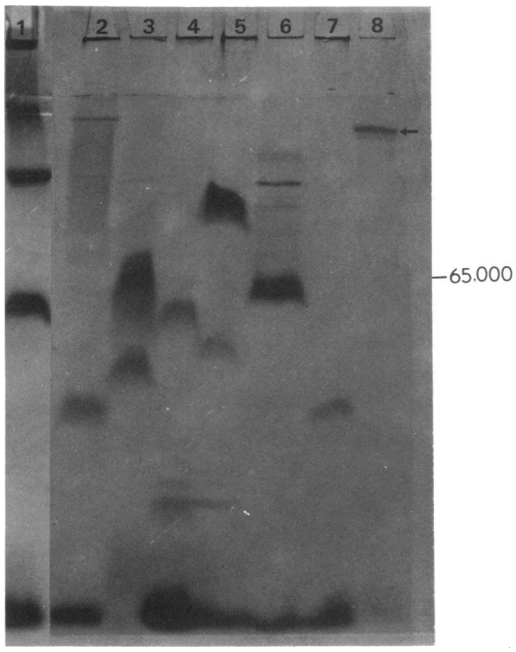

FIG. 7. *Gradient acrylamide-SDS gel electrophoretic separation of total protein from a series of motile (gliding) and nonmotile cyanobacteria stained with periodic acid-Schiff reagent. The 7 to 20% gels with a 5% stacking layer were prepared as described in the text. Lanes 1 through 7 each contain 100 μg of protein from the following strains: (1) Phormidium sp.; (2) A. halophytica; (3) G. violaceus; (4) Synechococcus sp. (UTEX 625); (5) Microcystis (PCC 7806); (6) O. limnetica; and (7) Dactylococcopsis sp. As a standard, lane 8 contains a total cell extract from H. salinarium strain 5, which has been shown to contain a 190,000-dalton glycoprotein (12) (arrow).*

lecular weights cannot be determined accurately by SDS-polyacrylamide gel electrophoresis (15), the initial estimate of 200,000 for the missing polypeptide was only approximate. The additional 160,000-dalton polypeptide which appeared in *mot-3* did not stain with periodic acid-Schiff reagent and might indicate that the mutation affected the polysaccharide portion of the glycoprotein.

On the basis of Coomassie blue staining, the glycoprotein from *A. halophytica* appeared to constitute between 0.25 and 0.5% of the wall protein. Peptidoglycan comprises the L-2 layer of the cyanobacterial cell wall and is primarily responsible for the characteristic wall strength (6). Since the mechanical strength of the wild type and of the *mot-3* mutant was identical (Fig. 6), the glycoprotein did not give the wall added rigidity, and the absence of the glycoprotein did not appear to affect the peptidoglycan in the wall.

Castenholtz (1) reported that nonmotile mutants of *O. terebriformis* which arise spontaneously ultimately become the dominant form in nutrient-rich liquid cultures. There are no apparent differences in the growth rates of the wild-type and the *mot-3* mutant of *A. halophytica* (data not shown), and even after several years of subculturing, the motile character remain the predominant type. There are slight physiological differences between the strains; however, *mot-3* contains more gas vesicles than the wild-type strain. The similarity of the pressure-collapse curves for the vesicles in the intact cells of the two strain types indicated that the characteristic collapse strength of the vesicles and the internal cell turgor pressure (19) were

probably identical.

Halfen and Castenholtz (8) provided evidence that a series of 6- to 9-nm-diameter fibrils located in the L-3 layer of the wall of *Oscillatoria princeps* is related to the motility mechanism. Cell wall fragments of *O. princeps* produced by sonication curled in a manner such that the fibrils in the L-3 layer which were originally oriented in a 60° right-hand helix around the filament were perpendicular to the longitudinal axis of the wall fragment. Fibrils were not visible in the wall fragments of *A. halophytica*, but similar curled fragments were a common phenomenon in both the wild type and the *mot-3* mutant (Fig. 4). The actual location of the glycoprotein is not known. Attempts to localize the proteins by selectively digesting the proteins on the outside layer of intact cells were unsuccessful (data not shown), either because the proteases were not active in the high salt concentrations required for cell viability or because cells were lysed under these conditions and the bulk of the proteins were digested.

Although it is not known whether *A. halophytica* contains the external fimbriae structures seen in several motile cyanobacteria (4, 11), such structures would not have remained attached to the wall fragments during their preparation and are not seen in the negatively stained wall fragments. However, the absence of differences in the protein pattern of other cell fractions indicates that if such structures are present, they are unaffected by the loss of the glycoprotein component.

In the series of cyanobacteria screened for the presence of high-molecular-weight (>65,000) glycoproteins, there was a good correlation between the presence of such proteins and motility. *G. violaceus* (14) was the only nonmotile organism to contain such a protein, and then only in small amounts. In the strains which show high rates of motility and do not form colonies on plates, the glycoprotein belonged to the abundant classes of proteins. For example, in cells of *Phormidium* sp., the glycoprotein constituted approximately 1% of the total protein. Determining whether proteins of this class are routinely involved directly in the motility mechanism or through interaction with other cellular structures (e.g., fimbriae) will require the isolation of additional mutants.

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