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Cytoplasmic Helical Structure Associated with *Acholeplasma laidlawii*

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A distinct spiral protein structure was found in three species of *Acholeplasma*, but was not found in the *Mycoplasma* species studied. The spirals, which are 14 nm in width and of variable length from 50 to 300 nm, are formed by a helical arrangement of 7-nm subunits. A rosette-like structure 45 nm in diameter also composed of 7-nm subunits was found in close association with the spirals and may be a taut in vivo form of the spiral. The electrophoretic profile in sodium dodecyl sulfate-polyacrylamide gels indicated that the spirals are composed of a predominant polypeptide with an apparent molecular weight of 100,000. No evidence can be found for inferring actin-like properties for this structure.

Several phenomena, such as the constriction of mycoplasma cells preceding cytoplasmic division (1), the reversible contraction of the plastic mycoplasma cells observed by microcinematography (1), the gliding motility of some mycoplasma species (1), and rotatory and flexional movement of the spiroplasmas (4, 19), suggest the presence of cytoskeletal structures in mycoplasmas. Support for this notion was provided by morphological and biochemical evidence that an actin-like protein is present in *Mycoplasma pneumoniae* (13) and *Spiroplasma citri* (C. Mouches et al., 3rd Conf. Intl. Org. Mycoplasmology, Custer, S. D., 1980, abstr. 100), a claim which is, however, disputed by Rodwell et al. (17).

During our experiments in search of an actin-like component in mycoplasmas, a spiral structure sensitive to proteolytic digestion was found in the cytoplasmic fraction of *Acholeplasma laidlawii*. The present report describes the purification of these structures and partial characterization of their morphological and biochemical properties.

MATERIALS AND METHODS

Growth conditions. *A. laidlawii*, *A. granularum*, and *A. axanthum* were grown in modified Edward medium (15) supplemented with 5% horse serum. The medium at an initial pH of 8.5 was inoculated with a 0.4% starter culture. The organisms were grown for 20 to 24 h at 37°C and harvested as previously described (15). *Mycoplasma capricolum* and *M. pulmonis* were grown under the same conditions except for an initial pH of 8.0 and an addition of 10% horse serum and 1% glucose to the *M. pulmonis* culture.

Fractionation of spiral structures. The procedure for isolation of the spiral structures is outlined in Fig. 1. Washed organisms were osmotically lysed by a 1:20 dilution in glass-distilled water preheated to 37°C. Phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 10 μg/ml, and the suspension was immediately cooled to 4°C. Membranes were separated by centrifugation (27,000 × *g* for 30 min at 4°C), and the supernatant (supernatant I) was centrifuged at 85,000 × *g* for 20 min at 4°C. Supernatant II was then centrifuged at 100,000 × *g* for 3 h at 4°C. Pellet III was suspended in imidazole buffer (3 mM, pH 7.5) containing 0.5 mM ATP, 0.7 mM 2-mercaptoethanol, 0.1 mM $CaCl_2$, and 10 μM PMSF. One milliliter of pellet III suspension was placed on top of a continuous sucrose gradient (5 to 40% [wt/wt]), which was prepared in imidazole buffer (100 mM, pH 7.5) containing 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid and 20 mM $MgCl_2$. The gradient was centrifuged at 95,000 × *g* (rotor SW27) for 16 h at 4°C. The gradient was unloaded, starting from the bottom of the test tube, by a Buchler peristaltic pump operating at speed 2. The fractions (1 ml) were assayed for protein and examined by electron microscopy and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Protein determination. Protein was estimated either by the Folin phenol reagent (9) with bovine serum albumin as a standard or by measuring the absorption at 290 nm.

Gel electrophoresis. Gel electrophoresis of proteins was carried out on 5.9% polyacrylamide gels containing 1% sodium dodecyl sulfate by the method of Porzio and Pearson (14). The molecular weight of the polypeptides was estimated by the use of standard markers: actin (43,000), myosin (200,000), and actin-binding protein (260,000).

DNase I inhibition. DNase I inhibition was assayed by the method of Lazarides and Lindbergh (8).

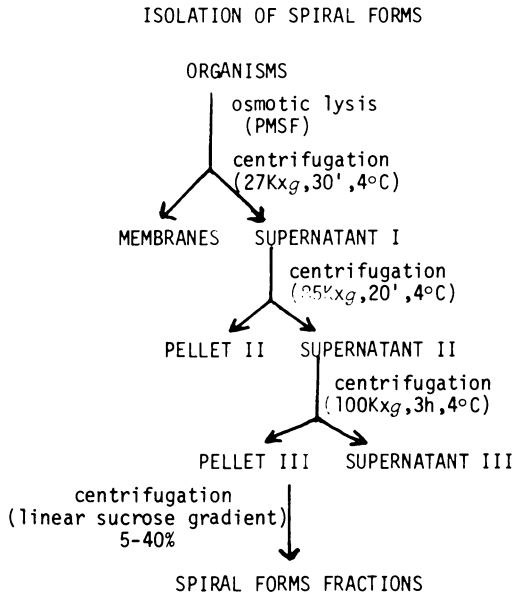

FIG. 1. *Flow diagram describing the fractionation procedure for obtaining the spiral structures.*

ATPase activation. The test for activation of heavy meromyosin-ATPase was carried out by the method of Muhlrad and Ferencz (12).

Ion-exchange chromatography. The isolation of actin-like molecules by ion-exchange chromatography on a DEAE-cellulose column was carried out by the method of Gordon et al. (6).

Electron microscopy. (i) Negative staining. A drop of the sample was placed on a carbon-coated collodion grid, and after 30 s it was removed by washing with 0.1 M NaCl. The grid was then stained with 5 to 10 drops of aqueous 1% uranyl acetate.

(ii) In situ lysis. Washed organisms were maintained in 0.25 M NaCl for a short time before a drop was placed on the grid. The grids were then washed with several drops of distilled water heated to 37°C and negatively stained as described above.

(iii) Decoration with heavy meromyosin. Decoration of the spiral structures and rabbit muscle actin with heavy meromysin was carried out by the procedure of Woodram et al. (21), and the preparations were examined by negative staining as described above.

(iv) Fixation and embedding. Cells of *A. laidlawii* were prefixed in situ by the method of Robertson et al. (16), fixed in 5% glutaraldehyde in sodium-phosphate buffer (0.1 M, pH 7.4) for 1 h at 4°C followed by washing, and maintained overnight in the same buffer. Cells were postfixed in 2% osmium tetroxide in the same buffer for 1 h at room temperature and washed with double distilled water. Cells were mixed with a small amount of 1% agar and stained en bloc with 1% uranyl acetate for 1 h. Cells were dehydrated in a graded series of acetone and embedded in Spurrs low viscosity medium (18). Sections were cut on a diamond knife. All of the electron microscope preparations were viewed, and the micrographs were recorded in a Philips EM400 operating at 80 kV.

(v) Optical diffraction. Optical diffractograms of selected helical structures were recorded on 35-mm film with the diffractometer designed by R. Josephs at the Weizmann Institute of Science.

RESULTS

Spiral structures in the cytoplasmic fraction. During our search for an actin-like protein in mycoplasma species, we used the procedure of Gordon et al. (6) for the isolation of *Acanthamoeba castellanii* actin, which employs an ion-exchange chromatography step on a DEAE-cellulose column. In the fraction which should contain the actin-like protein, we observed spiral-like structures in negatively stained preparations viewed in the electron microscope (Fig. 2). The possibility that these helical structures were formed during the chromatography procedure was ruled out as the structures were also observed in the cytoplasmic fraction obtained by osmotic shock (data not shown). However, unless special precautions were taken to prevent the action of proteolytic enzymes after the cell lysis, there was no evidence of these structures after 2 h at room temperature. The addition of 10 μg of PMSF per ml inhibited the proteolytic

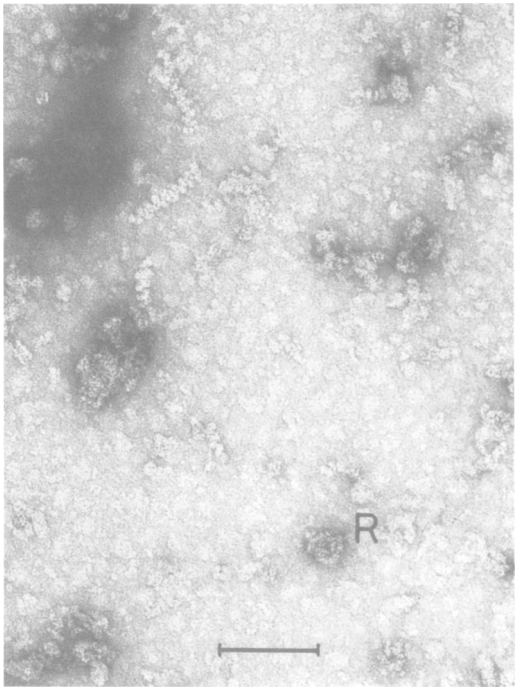

FIG. 2. *Negatively stained preparation of a typical field from the crude cell extract of A. laidlawii after fractionation on a DEAE-cellulose column. In addition to the spirals, structures similar in appearance to rosettes (R) are also noticeable. Magnification, ×137,500. Scale equals 0.1 μm.*

activity, consequently maintaining the helical structures intact.

We considered the possibility that these structures might be a postlysis polymerization artifact and sought to answer this question by two means. The first was to create conditions whereby the *Acholeplasma* cells could be lysed directly on the microscope grid, since we could reasonably presume that if the helical aggregates were found under these conditions, they were present in the cell. After in situ lysis the helical aggregates were indeed found in close proximity to the cell remnants (Fig. 3). Cells were found in various degrees of lysis, but in most cases clusters of strongly staining material were found in close association with cell remnants. Close examination of the clusters revealed that they consist of intertwining spirals which have a poor definition. Where the spirals have become detached from the clusters, they are seen in much clearer detail. Our second approach was to examine cells after thin sectioning to see whether the aggregates could be directly detected within the cell. This approach did not yield any conclusive results, but it is reasonable to assume that the very small diameter of the helical aggregates (14 nm) would make it difficult to distinguish them in thin section. In addition, the helices would have to be sectioned with a sufficient number of subunits along their long axis for a helix to be visible in thin section.

Purification of the spiral structures. When the lysate was fractionated as described in Fig. 1, the spiral structures were found concentrated in sucrose density gradient fractions 6 to 10. Figure 4 is a typical field of the spirals

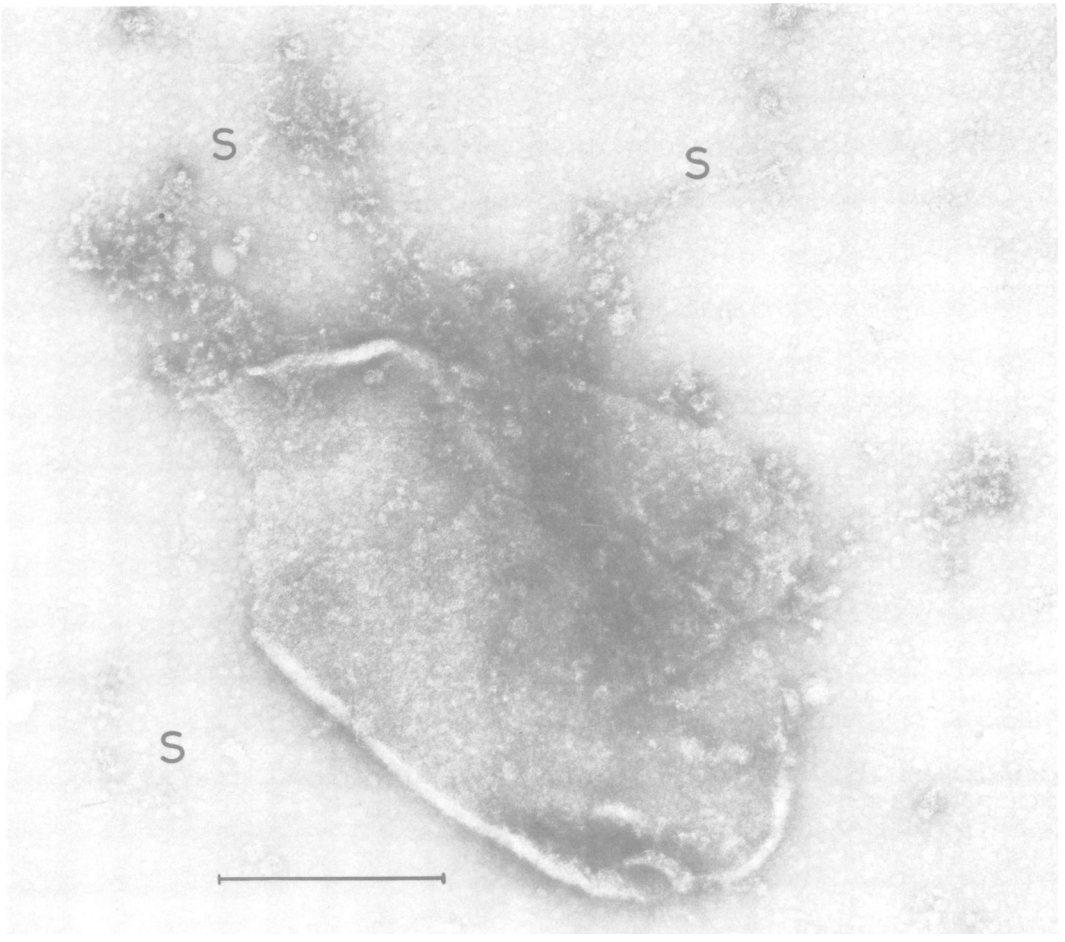

FIG. 3. *In situ lysis. Negatively stained membrane fragment from a cell of A. laidlawii lysed on the grid with distilled water heated to 37°C. Note the spirals (S) in clumps close to the cell fragment and their increasing clarity the farther they are from the cell fragment. Rosette-like structures are also evident. Magnification, ×60,000. Scale equals 0.5 μm.*

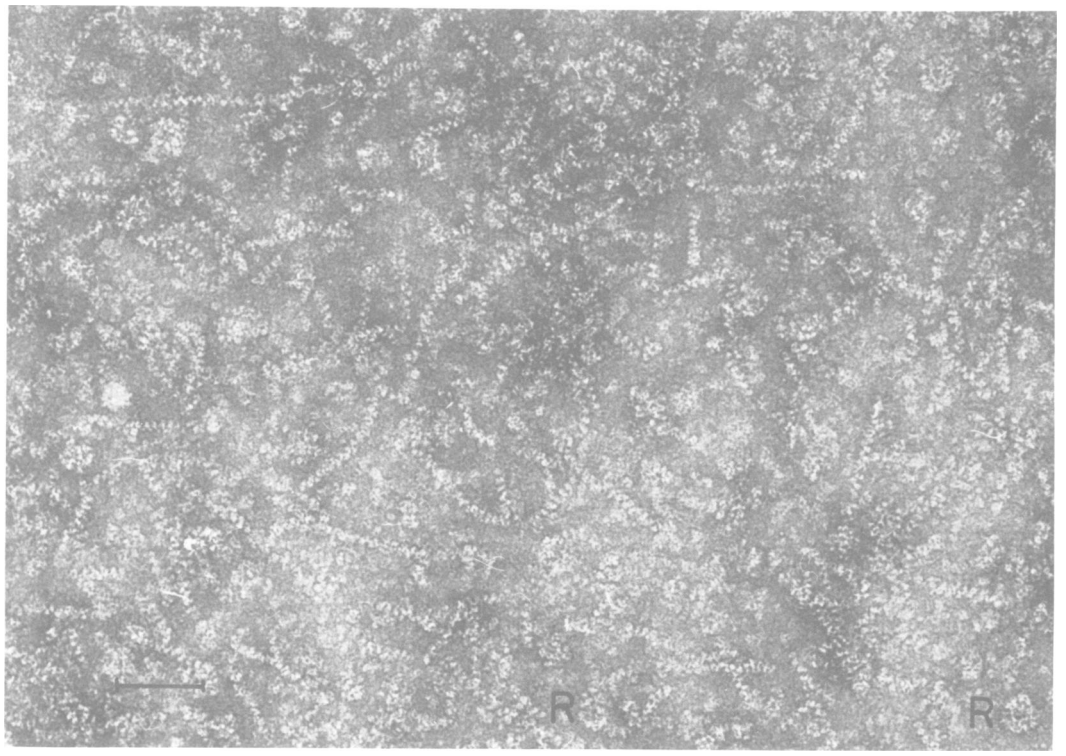

FIG. 4. *Electron micrograph of a negatively stained field from the sucrose density gradient fraction containing the highest concentration of spirals isolated from A. laidlawii. Note the variable length of the spirals. Occasional rosettes (R) are also evident. Magnification, ×110,000. Scale equals 0.1 μm.*

which are of constant width of 14 nm but are of variable length from 50 to 300 nm. The spirals are rigid over short distances, but many are also seen to curve in a semicircular fashion. When examined at higher magnification, the spirals were seen to be helical in character and were made up of 7-nm subunits. Figure 5 shows a gallery of the helices of different lengths with each exhibiting the same basic features.

To characterize the helical parameters of the spirals, selected images were analyzed by optical diffraction. The dominant feature of the diffraction pattern (Fig. 6) is a pair of off-meridional reflections on the first layer line. From the height of these reflections the pitch of the helix was calculated to be 9 nm. No meridional reflection could be seen clearly in the images selected, and we could not obtain spirals sufficiently long to give diffraction patterns showing the helical repeat distance.

Rosette-like structures. Examination by electron microscopy of the prominent yellow band in fraction 4 of the sucrose gradient showed mostly distinct circular rosette-like structures which were of almost constant diameter (45 nm; Fig. 7). The fields of rosettes were interspersed

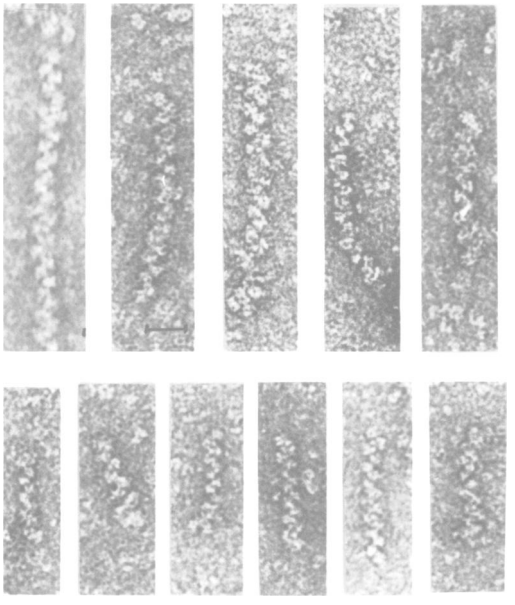

FIG. 5. *Gallery of helical aggregates isolated from A. laidlawii showing the variable lengths of the spirals. Magnification, ×214,000. Scale equals 50 nm.*

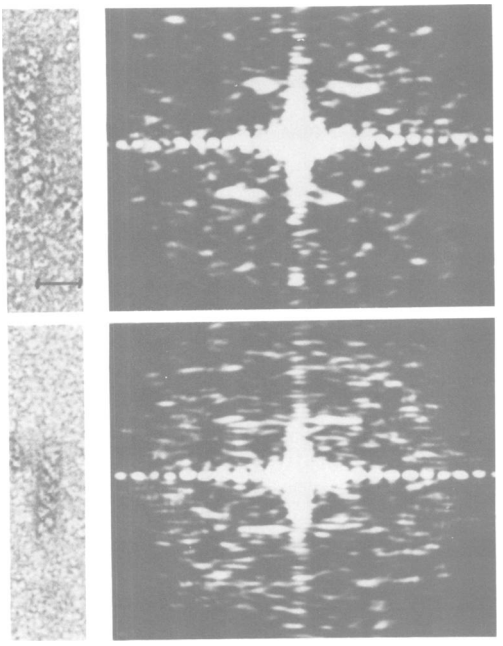

FIG. 6. *Optical diffraction of spirals. Electron micrographs and corresponding optical diffraction patterns of negatively stained helical aggregates from A. laidlawii. The distance to the first layer line corresponds to 9 nm. Magnification, ×214,000. Scale equals 50 nm.*

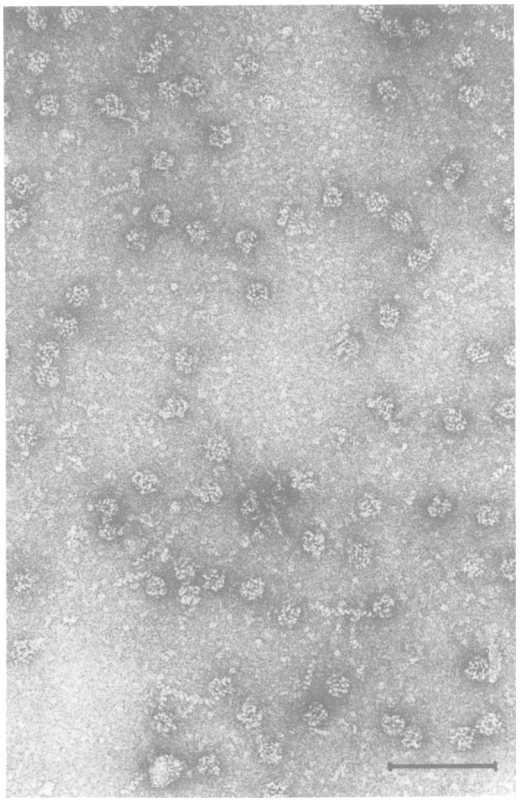

FIG. 7. *Micrograph of a negatively stained field of rosettes showing the uniformity of size of these structures. The field also shows a number of spirals. Magnification, ×62,700. Scale equals 0.5 μm.*

with a relatively low concentration of spiral structures. At higher magnification (Fig. 8), the rosettes were seen to have a substructure showing subunits of 7 nm in diameter. Some of the rosettes exhibited a strongly staining central region. A comparison of the circumference of the rosettes (ca. 97 nm) with the length of a number of the spirals (78 nm) seen in the field in Fig. 7 showed the latter to be slightly shorter but in the same size range, nevertheless. The circumference of the rosette was calculated from the diameter of the particle less twice the width of one subunit.

Biochemical properties. The electrophoretic profile of fractions obtained at various steps of the purification (Fig. 9, gels 1 through 4) indicated that the fraction enriched with the rosettes was composed of a large number of polypeptides (Fig. 9, gel 3). This fraction was also enriched with the polypeptide with an apparent molecular weight of about 100,000, which was the predominant single polypeptide in the spiral-rich fraction (Fig. 9, gel 4). Our attempts to decorate the spiral structures with heavy meromyosin to show their similarity to actin were negative, whereas rabbit muscle actin controls were positive. In addition, the test for heavy meromyosin-ATPase activation by the spiral fraction also proved negative. The fractions with high concentrations of spirals showed no inhibition of DNase I activity.

Spiral structures in other mycoplasmas. To determine whether or not the appearance of the spirals is unique to *A. laidlawii*, we examined two additional species of *Acholeplasma*, *A. axanthum* and *A. granularum*, and two species of *Mycoplasma*, *M. pulmonis* and *M. capricolum*. Spirals were easily detected in the two additional *Acholeplasma* lysates examined, whereas no spirals were found in the two *Mycoplasma* species tested. The spirals thus appear to be unique to acholeplasmas.

DISCUSSION

We have considered the possibility of a cytoskeletal role for the spiral structures described in this study. Their helical nature, variable length and, most likely, their subunit composition could support this notion. The existence of rosettes may be evidence for a compact form of the spirals which relax to form filaments when

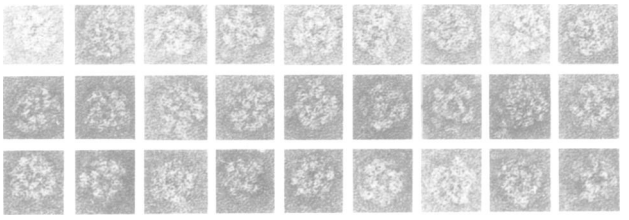

FIG. 8. *Gallery of rosettes at higher magnification showing details of their substructure. Some particles show a distinct central circular structure. No clear subunit symmetry is apparent in the particle. Magnification, ×135,000. Scale equals 0.1 μm.*

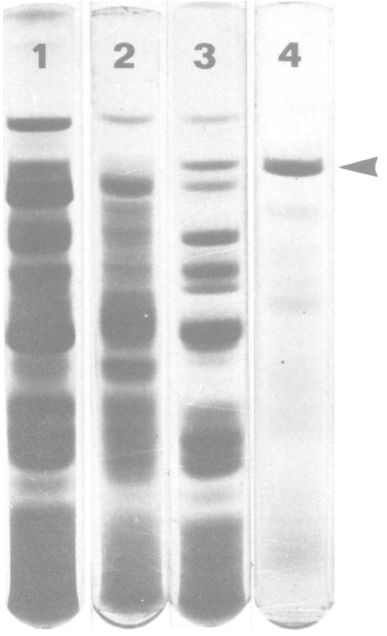

FIG. 9. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of the spiral structures of A. laidlawii. 1, Supernatant II from which pellet III was subsequently derived; 2, pellet III which was the source material for the sucrose density gradient; 3, yellow fraction from the sucrose density gradient; 4, sucrose density gradient fraction containing the highest concentration of spirals.*

the cell is lysed. A number of factors, however, weigh against inferring actin-like properties for the spirals. (i) They have a molecular weight of 100,000 compared to a molecular weight of 43,000 for actin. (ii) There is no direct correlation between DNase I inhibition and the presence of these structures. (iii) The DNase I inhibitory factor which was found in *A. laidlawii* (I. Peleg, A. Muhlrad, and I. Kahane, unpublished data) is stable at room temperature, whereas the helical structures are not. (iv) The spirals could not be decorated with heavy meromyosin. (v) The spirals could not activate heavy meromyosin-ATPase. (vi) Cytochalasin B has no effect on *A. laidlawii* (11).

We were initially worried that we might be dealing with a postlysis polymerization artifact, but the in situ lysis experiments strongly supported the fact that the spirals existed as such in the cell. It is tempting to consider the rosettes as a taut form of the spirals, although other proteins may be associated in the rosette, as suggested by the complex electrophoretic profile of the fraction. It is also difficult to decide whether the spirals are formed from the rosettes or the opposite. The constant diameter of the rosettes would favor the former, allowing the variable length of the spirals to be related to a measure of postlysis polymerization.

We cannot exclude the possibility that we may be dealing with a virus-related structure. However, the helical structure does not resemble any known mycoplasma virus (3).

We have also considered the possibility that the helical structures found in the acholeplasmas might bear a relationship to the helical ribosomes described in *Mycoplasma gallisepticum* by Maniloff (10). However, a helix diameter of only 14 nm is too small for the known size of 70S ribosomes. Clark et al. (2) have recently described a helical array of the 30S ribosomal subunits from *Escherichia coli*, which displays two different projections, one with a width of 32 nm and one with a width of 18 nm. In our case, all of the projections have the same width of 14 nm and are therefore not similar to the ribbons of ribosomal subunits.

Structures similar to those described in the present study have been seen in lysates of two other *Acholeplasma* species (R. M. Cole, personal communication). Spiral forms bearing some resemblance to those in the acholeplasmas were also found in *E. coli* B and other bacteria (J. P. Rosenbusch and R. M. Cole, personal communication). There is no evidence as to their function in the bacterial cell. The fact that the spirals are found only among members of the *Acholeplasmataceae* is further evidence of the

distinct taxonomic standing of this family within the *Mycoplasmatales*.

Finally, the spiral structures might also possibly be considered a polymorphic form of an enzyme similar to the cable forms of glutamic dehydrogenase (7) and glutamine synthetase (5), or the polydisperse rods of pyridine nucleotide transhydrogenase (20). We are currently investigating this possibility.

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