

Growth and Division of *Spiroplasma citri*: Elongation of Elementary Helices

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The smallest viable cell of *Spiroplasma citri* is a two-turn helix (elementary helix). This elementary helix grows into longer parental cells, which then divide by constriction. The helical morphology is conserved during this process. The growth pattern of *S. citri* membranes has been investigated by different methods of membrane labeling. When labeling is done with specific antibodies, a diffuse growth of the membrane is observed. On the contrary, pulse-labeling of the membrane with tritiated amino acids reveals a polar growth of the organism. Finally, labeling of oxydo reduction sites with potassium tellurite also indicates a polarity in the organism. These results are discussed, and a scheme for spiroplasma growth is proposed.

We have shown previously (5) that the helical mollicute *Spiroplasma citri* grows from short elementary helices into longer parental helices. The latter divide by constriction, producing elementary helices. During this process the helical morphology of the organism is conserved, and elementary helices have been shown to display a morphological polarity: one end of the helix is tapered and the other is blunt.

Membrane synthesis during growth and division may be an important parameter for maintenance of morphology and polarity in *S. citri* cells. Quinlan and Maniloff (8) reported that in *Mycoplasma gallisepticum* the DNA growing point is attached to the membrane. This indicates that, as for bacteria, membrane synthesis could act to segregate DNA genomes during division of the organism. It was therefore of interest to determine the growth pattern of *S. citri* membranes (diffuse growth or specific growing zones) in view of its role in morphology and DNA replication. For bacteria, studies of membrane elongation have yielded results varying with the method used to label the cell surface. Rapid movements of proteins and lipids in the membrane must be taken into consideration in evaluating the type of labeling observed (for a review see reference 6). For this reason, we chose three different methods of membrane labeling to study the growth pattern of *S. citri* membranes. In method 1, the entire surface of the organism was coated with specific antibodies; the cells were then allowed to grow in antibody-free medium. New membrane areas would be reported to have a lesser density of antibodies. In method 2, potassium tellurite was used to label newly formed oxydo reduction sites in the membrane. In method 3, newly synthesized membrane proteins were pulse-labeled with tritiated amino acids.

MATERIALS AND METHODS

***S. citri*.** The Morocco strain R8A2 (ATCC 27556), subcultured more than 500 times, was grown at 32°C in BSR medium (1) buffered with 0.06 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (BSRH medium). This strain was grown in BSRH medium containing fetal calf serum instead of horse serum for experiments in which antibodies against *S. citri* were used.

Visualization of cell surface-fixed antibodies by ferritin

labeling. Antibodies produced by injection of *S. citri* proteins into rabbits as described previously (11) were added to an early-log-phase culture grown in BSRH medium (containing fetal calf serum) at a concentration close to the deformation titer (12). After 2 h at 32°C, the culture was centrifuged for 20 min at 16,000 × *g*, and the pellet was washed with and resuspended in antibody-free medium. Samples of the culture were taken before and after centrifugation and at 60-min intervals thereafter until late log phase was reached (60 h). The samples were fixed with glutaraldehyde in collidine buffer (1% final) for 15 min.

The antibodies fixed on the membrane of *S. citri* were revealed by incubating 14 μl of fixed culture with 7 μl of ferritin-conjugated goat anti-rabbit immunoglobulin G (IgG) (lot no. 5423; Miles Laboratories, Inc.) (precipitating titration, 1/640) for 2 h. The organisms were then observed in an electron microscope by the transfer technique (5). Controls were performed by incubation of ferritin-conjugated goat anti-rabbit IgGs with a culture of *S. citri* which had not been incubated with specific antibodies.

The growth curve of the organism was determined by counting the number of organisms by the transfer technique as already described (5).

Labeling of oxydo reduction sites of *S. citri* with tellurium. The method was essentially that described by Ryter (10) for bacteria. A 5% solution of potassium tellurite was prepared in water, and the pH was lowered to pH 9 by addition of 0.1 N HCl. This tellurite solution was added to an early-log-phase culture of *S. citri* to a final concentration of 0.05%. The organisms were incubated at 32°C, and the labeled sites were determined after 2, 4, and 6 h by means of the transfer technique.

Labeling of *S. citri* with tritiated amino acids. A mixture of tritiated amino acids (Amersham Corp.) (specific activity > 40 Ci/mmol) was added to 20 ml of a 38-h-old culture to give a final concentration of 50 μCi/ml. The radioactivity incorporated by the organisms was determined on 0.3-ml samples after 30 min, 1, 2.5, and 5 h of growth at 32°C. The samples were treated with 15% trichloroacetic acid; the precipitates were washed six times with 5 ml of 10% trichloroacetic acid, then with 5 ml (vol/vol) of an ethanol-ether mixture, and finally twice with 5 ml of petrol ether. The radioactivity was counted in a scintillation spectrometer (Intertechnique SL30). Autoradiography with Ilford L4 emulsion was per-

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formed on samples taken at the same time and submitted to the transfer technique. Membrane purification (see below) was applied to organisms after the 1-h incorporation period. Radioactivity of the membrane fraction was determined as below.

***S. citri* membrane purification.** The organisms in 20 ml of culture were centrifuged for 20 min at $20,000 \times g$, resuspended in 20 ml of 0.1 M $MgCl_2$, and centrifuged for 20 min at $20,000 \times g$. The pellet was resuspended in 1 ml of β -buffer diluted 20-fold (β -buffer is 5 mM NaCl, 50 mM Tris base, 10 mM β -mercaptoethanol, pH 7.5). A 10- μ l portion of DNase (1 mg/ml) was added, and the mixture was incubated for 10 min at 37°C. A 40-ml portion of β -buffer containing 10 mM EDTA was added. The membrane fraction was obtained by centrifugation for 20 min at $35,000 \times g$. The pellet was

washed twice with 30 ml of β -buffer plus EDTA and resuspended in 1 ml of β -buffer. The radioactivity present in the three final supernatants and the radioactivity present in the final resuspended membrane pellet were determined on 0.3-ml samples, after addition of 40 μ l of bovine serum albumin at 10 mg/ml and precipitation with 15% trichloroacetic acid as indicated above.

RESULTS

Antibody labeling. When *S. citri* was incubated for 2 h in the presence of specific antibodies, a uniform layer of immunoglobulins adsorbed on the surface of the organism, as witnessed by the labeling obtained with ferritin-conjugated goat anti-rabbit IgGs (Fig. 1A). Controls with goat IgGs

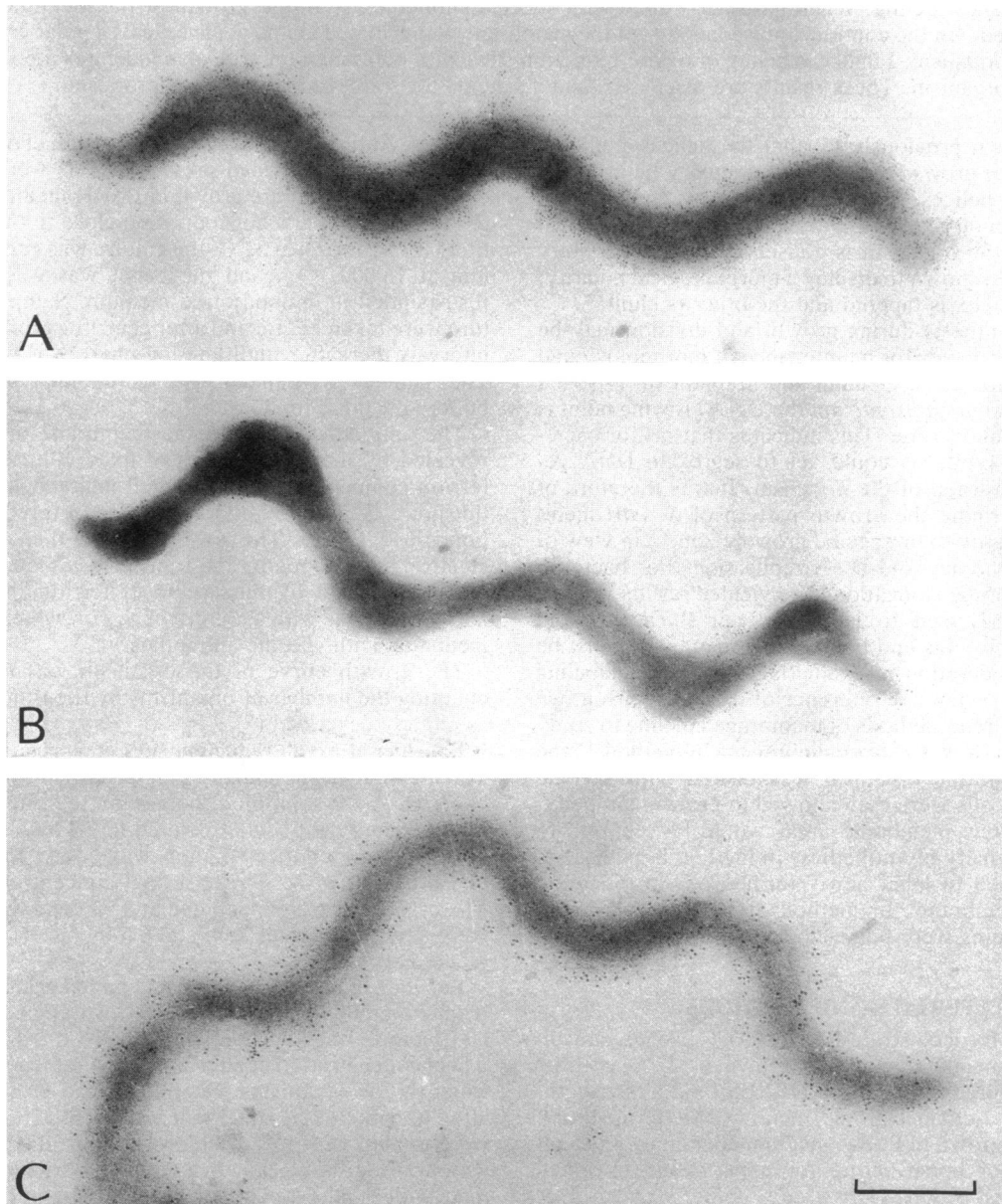


FIG. 1. Ferritin labeling assays of *S. citri* cells. (A) Ferritin labeling immediately after coating of *S. citri* with specific antibodies. (B) After coating with specific antibodies, the organisms were allowed to grow for 6 h in antibody-free medium before ferritin labeling. (C) Same as B but with ferritin labeling after 24 h in antibody-free medium containing 45 μ g of tetracycline per ml. Bar, 0.5 μ m.

alone without prior coating with specific antibodies resulted in no labeling of the cell surface. Under our conditions, when antibody-coated *S. citri* cells were transferred into antibody-free medium, the organisms did grow and divide as indicated in Fig. 2, in which the growth curves of a control culture (●) and of the culture in the presence of antibodies (■) have been determined. The newly synthesized membrane should be devoid of antibodies and, therefore, of ferritin labeling. When organisms first coated with specific antibodies were placed for various times of growth in antibody-free medium, the layer of ferritin began to decrease only after 4 h but in a homogeneous fashion over the entire surface of the helix. The ferritin layer observed after 6 h of growth in antibody-free medium is illustrated in Fig. 1B. No specific unlabeled zones could be observed. In this experiment, the time the organisms grew in IgG-free medium was relatively long (4 to 6 h), and IgGs could have been gradually released from the helices into the medium. To test this possibility, 75 µg of tetracycline was added to 1 ml of the IgG-free culture. This resulted in a growth inhibition of the antibody-tagged organism as shown in Fig. 2 (curve Δ). At 24 h after transfer to the antibody-free medium, the ferritin layer on the cell surface (Fig. 1C) was similar to that observed on the control (Fig. 1A). Therefore, when attached to the surface of *S. citri*, specific antibodies remain tagged to the cell for at least 24 h; and the decrease of label observed from Fig. 1A to B must be a consequence of an elongation of the organisms.

Labeling of oxydo reduction sites of *S. citri* with potassium tellurite. The reduction of potassium tellurite leads to the formation of electron-dense tellurium crystals made easily visible by electron microscopy. Ryter (10) studied the growth of *Bacillus subtilis* by using potassium tellurite. The entire membrane of *B. subtilis* was labeled in 20 min, and after transfer to potassium tellurite-free medium localized growing zones could be detected. When added to an *S. citri* culture, potassium tellurite is highly toxic, and the maximum concentration of potassium tellurite that can be added is 0.5 mg/ml. A higher dose results in destruction of the helix and death of the organism. With a concentration of 0.5 mg/ml, 5 to 6 h are necessary to observe label in *S. citri*. Under such conditions, about 20% of the organisms contain tellurium

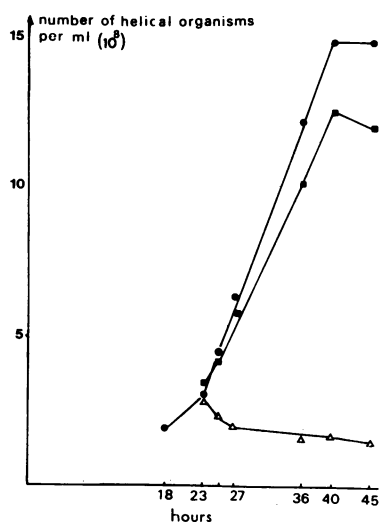


FIG. 2. Growth of *S. citri* as determined by the transfer technique: ●, in BSRH medium (control); ■, in BSRH medium plus IgG; Δ, in BSRH medium plus IgG plus 75 µg of tetracycline per ml.



FIG. 3. Electron micrograph of *S. citri* grown for 5 h in the presence of a 5-mg/ml solution of potassium tellurite. (A) Elementary helix, (B) parental helix. Bar, 0.5 µm.

crystals. In 99% of the tellurium-containing organisms, the crystals were localized at the blunt end only (Fig. 3A). In a few organisms, it was possible to observe tellurium crystals at two different sites: at the blunt end and at the middle of the helix or, more precisely, at one end of the future helix (Fig. 3B). Thus, oxydo reduction sites in *S. citri* seem to be active only at one end of the organism and occasionally at cell division points, revealing a polarity of the organism.

Growth pattern of *S. citri* as revealed by short labeling with tritiated amino acids. To observe newly synthesized membranes produced during shorter growth periods than those

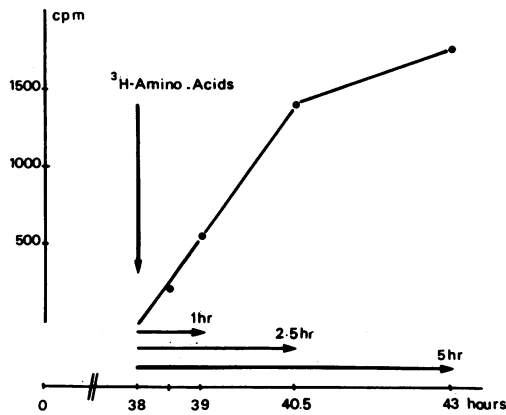


FIG. 4. Incorporation of tritiated amino acids in *S. citri* proteins as a function of time.

used in the previous experiments, pulse-labeling of *S. citri* with tritiated amino acids was performed with a mid-log-phase culture (38 h of growth). The radioactivity incorporated in *S. citri* proteins was determined after 30 min, 1, 2.5, and 5 h (Fig. 4). The relatively low amount of radioactivity incorporated was the result of the high dilution of the radioactive amino acids with the unlabeled amino acids present in the culture medium.

Observation of elementary helices after high-resolution autoradiography revealed that some (19%) were already labeled after 30 min of growth. The percentage of labeled elementary helices increased with time and after 5 h reached 63% (Table 1). At that time, elementary helices showed a more or less uniform distribution of silver grain over the surface (Fig. 5).

When observed after a 1-h pulse, elementary helices and four-turn parental helices showed a more specific localization of labeling (Fig. 6 and Table 2). A large proportion (73%) of elementary helices were labeled at one end, but some (19%) had the label in the middle of the helix (Fig. 6A). Only a few organisms (8%) showed silver grains at two sites (Table 2 and Fig. 6B). A closer examination of elementary helices labeled in the middle of the helix revealed that most of these showed the beginning of a constriction (Fig. 6C). Therefore these helices, in spite of their short size, do not correspond to real elementary helices but are prematurely dividing parental forms. The same distribution of labeling was observed in parental helices: most of them (69%) were labeled at one end (Table 2 and Fig. 6D) when no constriction was present on the organism. Other organisms (27%) were labeled in the middle of the helix (Fig. 6E), and this labeling probably occurred at a site of future constriction because a division at that site will liberate two-turn helices. When a constriction was already present, 46% of the orga-

TABLE 1. Labeling of *S. citri* with tritiated amino acids: number of labeled helices as a function of time

Time (h)	Total no. of elementary helices observed (transfer technique)	% Labeled helices	% Unlabeled helices
0.5	269	19	81
1.0	376	36	64
2.5	260	51	49
5.0	300	63	37

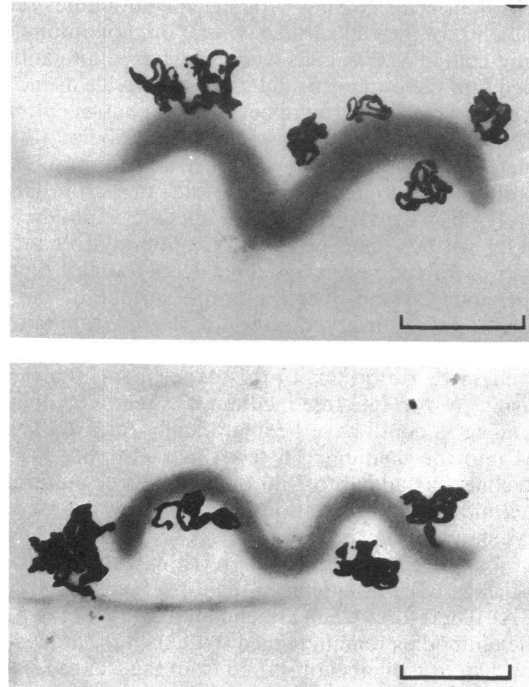


FIG. 5. Labeling of *S. citri* with tritiated amino acids: autoradiography of elementary helices after the 5-h pulse. Bar, 0.5 μ m.

nisms were labeled at the constriction level, and 43% were labeled at one end of the helix.

The amount of the radioactivity incorporated in membrane proteins was determined for the 1-h pulse (Table 3). Half of the radioactivity was incorporated into membrane proteins (18,981 cpm), and the other half was incorporated into cytoplasmic proteins (20,380 cpm). Therefore, the label observed after autoradiography of the organism reflects the label in both cytoplasmic and membrane proteins.

DISCUSSION

These experiments show that, as for bacteria, different results are obtained concerning the growth of *S. citri* according to the method used to reveal growing zones. The small size of the organism and the absence of a cell wall limit the number of useful methods.

From the data on labeling with tritiated amino acids, a scheme for *S. citri* growth can be deduced (Fig. 7A). An elementary helix with one blunt and one tapered end (a) develops into an elementary helix with two blunt ends (b). Growth will start at one end as represented by the dark area on the organism (c). During the same time, parental helices which are ready to divide (A) grow around the constriction

TABLE 2. Labeling of *S. citri* with tritiated amino acids: radioactivity distribution after a 1-h pulse

Site of labeling	% Labeled helices			
	Elementary	Four-turn parental		
		Without constriction	With constriction	
One end only	73 { Blunt end 63 Tapered end 37	69	43	
Middle only	19	27	46	
One end and middle	6	4	6	
Both ends	2	0	5	

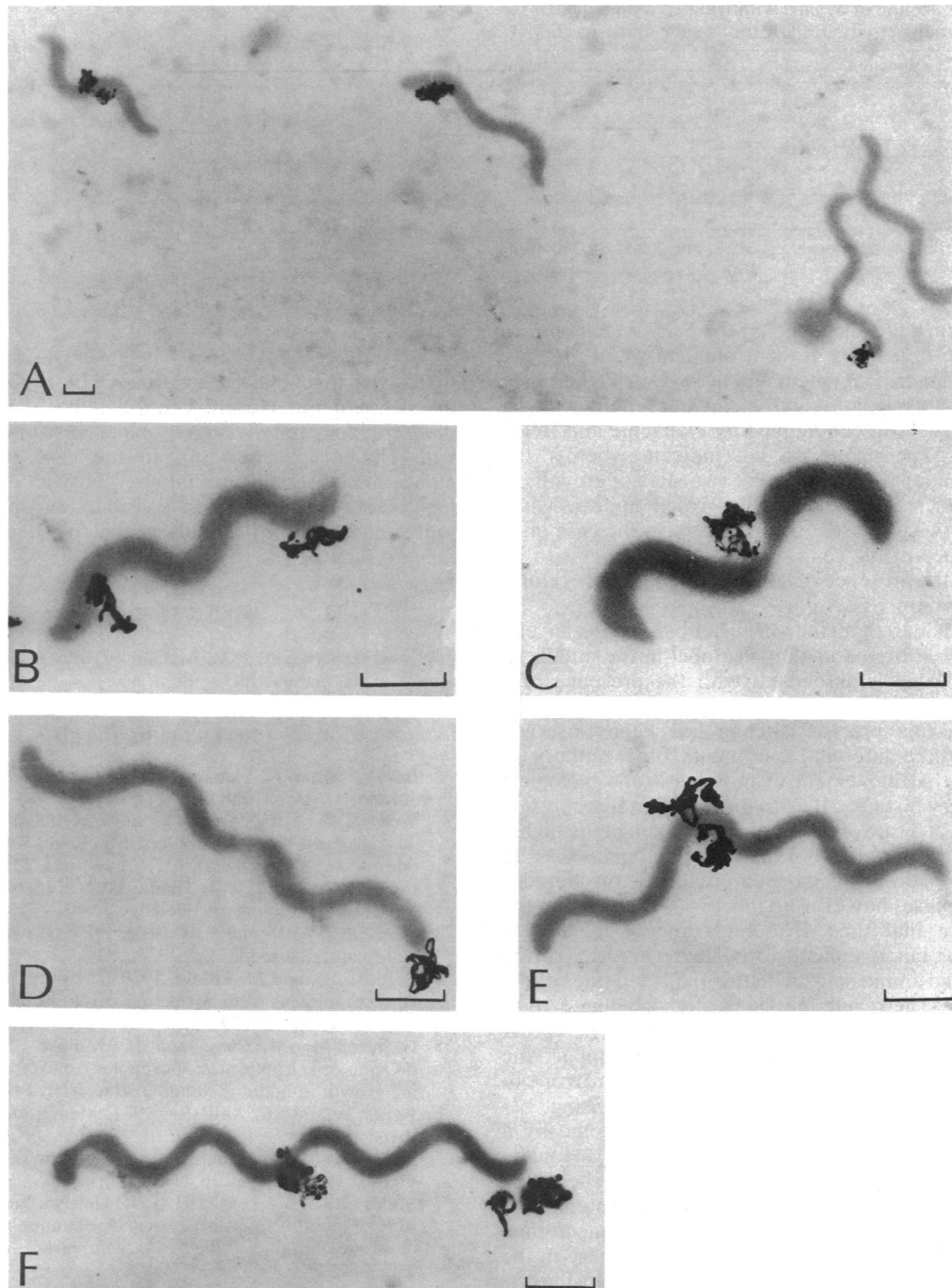


FIG. 6. Labeling of *S. citri* with tritiated amino acids: autoradiography of elementary (A and B) and parental helices (D, E, and F) after a 1-h pulse. (C) Elementary helix with constriction. Bar, 0.5 μ m.

(B) to yield two elementary helices with one blunt end and one labeled, tapered end (C). This tapered end may turn into a blunt end (C1). In agreement with the radioactive labeling data, the percentage of elementary helices with two blunt ends labeled at one end was 63%, whereas the percentage of helices with a labeled tapered end was 37% (Table 2). The presence of elementary or parental helices labeled at two sites is explained in Fig. 7B. It implies that an elementary helix already labeled at one end grows into a four-turn parental helix and then becomes labeled in the middle at the

place where a constriction will occur. This constriction will lead to the formation of two elementary helices, one with label at both ends and one with label at only one end. These events are probably rare during a 1-h pulse because such a pulse represents a short period of time in comparison with the doubling time of a culture, which is about 6 h. Hence, elementary helices with label at two sites are rare events after a 1-h pulse, and the percentage of such helices was between 2 and 10% (Table 2).

This growth model is very similar to the unit cell model

TABLE 3. Labeling of *S. citri* with tritiated amino acids: distribution of radioactivity in the various subcellular fractions

Fraction	Radioactivity (cpm)
Organism	54,774
Supernatant ^a	
1	11,200
2	4,560
3	4,620
Membrane pellet	18,981

^a Total radioactivity for supernatants 1, 2, and 3 was 20,380 cpm.

described by Donachie and Begg (3) for *Escherichia coli*. In their model, the organisms grow at one site when they are less than a certain critical length and at two sites when they are more than this length. Our elementary helix can be compared with the unit cell defined by Donachie and Begg. However, our experiments do not indicate whether the growth is bidirectional when we have two sites of growth. In the unit cell model, the second site of growth appears when *E. coli* reaches twice the size of a unit cell. In the case of *S. citri*, the presence of the central growing zone seems to be correlated with the presence of a constriction and, therefore, with the division process.

Organisms smaller than four-turn helices (twice an elementary helix) can be found showing label in the middle of the helix. This label is associated with the presence of a constriction.

Even though this precise labeling has been observed consistently in three different experiments, other authors (4, 7) have shown that movement of proteins in the biological membrane is very fast (1×10^{-10} cm/s) and that lipopolysaccharides diffuse at a rate of 5×10^{-13} cm/s, even in the highly viscous outer membrane of *Salmonella typhimurium*. In such conditions, 30 min of labeling could be considered a long time for a pulse, however no label was observed on the organism before that time. The real time of labeling is certainly shorter but is difficult to evaluate because of the dilution of labeled amino acids with the unlabeled ones of the growth medium. The results obtained after labeling *S. citri* with potassium tellurite agree with the hypothesis of a polar growth of elementary helices, and they also support the finding of morphological polarity. The diffuse growth observed when antibodies were used may be surprising, because an equatorial growing zone has been detected by a similar technique with *Streptococcus pyogenes* (2). However, in that case, the cell wall of the organism was labeled. In 1970, Frye and Edidin (4) demonstrated that proteins and lipids are constantly redistributed all through the membrane because of lateral movements. If this is also the case in the presence of antibodies, it would explain the homogeneous distribution of antibodies over the entire surface of the organism. Similar experiments with pulse-chase labeling of *S. citri* membranes with tritiated palmitic acid also resulted in a random distribution of the label (M. Garnier, unpublished data). No experiments have been made concerning the growth pattern of other spiroplasma or mycoplasma membranes, however Rodwell et al. (9) compared the labeling of lipids of *Mycoplasma mycoides* in the part of the membrane to which DNA is attached with the labeling of lipids in the entire membrane and found that there was no evidence for local lipid synthesis in this region of the membrane. The authors estimated that these experiments were inconclusive because of the high probability of lipid redistribution in the membrane.

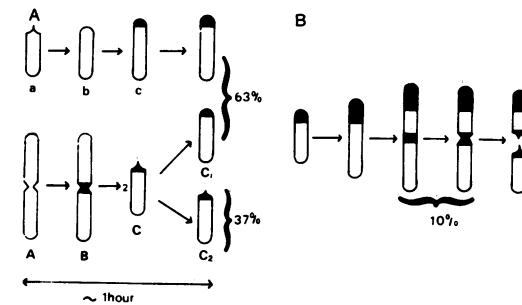


FIG. 7. Scheme for *S. citri* growth.

In most mycoplasma species, DNA replication and cell division are not closely coordinated (9), however in *M. gallisepticum*, cell division by binary fission is synchronous with DNA replication (8). In the case of *S. citri*, the elongation observed around the constriction zone, if real (and before the constriction is visible), may play a role in the segregation of DNA strands in the two newly formed elementary helices.

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