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Biology of *Azospirillum*-Sugarcane Association: Enhancement of Nitrogenase Activity†

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Azospirillum brasilense was reisolated from associations with callus tissue cultures of sugarcane and compared with stock cultures of the inoculated bacterium and related strains. Although the reisolate had a growth rate similar to stock cultures, it exhibited a severalfold increase in maximum specific activity of nitrogenase. The reisolate and the parent culture had similar ultrastructure. The general ultrastructure of *Azospirillum* is described. The bacterium was capsulated when grown on nitrogen-free nutrient agar plates and on callus, but was not capsulated when growing in a subsurface zone in N-free semisolid nutrient agar, except rarely in aging cultures. Capsulation may be a protective mechanism against unfavorable pO_2 under dinitrogen-fixing conditions. Pleomorphism occurred in capsulated forms, and the ultrastructure of these forms is described.

Azospirillum brasilense (*Spirillum lipoferum*, 29) was originally isolated from the rhizosphere of the tropical grass *Digitaria decumbens* (12) and has been suggested as a potential source of biologically fixed nitrogen for some nonleguminous agronomic crops (13, 26, 27). There is no consistent evidence that this bacterium contributes significant amounts of nitrogen to plants with which it is associated (1, 9, 30), despite some reports of enhanced plant growth in field trials (8, 26). A more thorough understanding of the interaction between this bacterium and higher plants under associative conditions could provide information on how and whether it may be used more effectively in reducing fertilizer demands for certain crops.

Stimulation of nitrogenase activity in *Azospirillum* grown in association with plant callus tissue cultures has been reported, and it has been suggested that the callus tissue provided organic acids that were utilized by the bacterium (10). We have recently described the establishment of long-term associations of *Azospirillum* with sugarcane tissue cultures and have demonstrated considerable nitrogenase activity in the associated cultures (33). An ultrastructural study of the associations revealed luxuriant growth of the bacteria on the surface and in the intercellular spaces of the callus tissue (4). The present paper describes the growth, nitrogenase activity (ARA), and the ultrastructure of the bacteria reisolated from the associated cultures of *Azospirillum*-sugarcane. Nitrogenase activity of the reisolated bacterium was shown to be appreciably, albeit temporarily, higher than that of the parent strain.

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MATERIALS AND METHODS

Abbreviations. The abbreviations used in this paper are as follows: ARA, acetylene reducing activity; LPS, lipopolysaccharide; PASH, periodic acid-silver hexamine; PHB, poly-β-hydroxybutyrate; and SNF, succinate nitrogen free.

Acetylene reduction. Sugarcane callus tissues were inoculated with *A. brasilense* strain 7 (ATCC 29145), and such associated callus cultures were maintained for over 18 months by subculture as previously described (33). Two experiments were performed to compare callus-associated bacteria with stock strains. In the first, bacteria were washed from callus pieces by shaking in sterile deionized water. This inoculum and that prepared to approximately equal optical density from stock Trypticase soy agar (BBL Microbiology Systems) slants of *A. brasilense* strains 7 and 13t were each implanted by loop into 12 screw-capped tubes (13 by 100 mm) containing 3 ml of SNF medium (0.05% agar; for formulation, see 31) and incubated at 35°C for 6 days. The screw caps on the tubes were replaced with serum stoppers, and 10% of the gas volume was replaced with pure acetylene. Ethylene production was measured 24 h later with a Varian 2400 gas chromatograph (31). The 12 tubes of each culture were then pooled, and the bacterial cells were concentrated and washed twice by centrifugation in sterile deionized water. The protein content of the pooled cells was measured by the fluorescamine method (5). The use of 0.05% agar in cultures used for measuring ARA was required to permit concentration of cells from multiple tubes for protein measurement. Comparative studies in SNF with either 0.3, 0.05, or 0.01% agar showed that growth and ARA in the 0.05% concentration was equal or superior to the other concentrations for all cultures used in this study (unpublished data).

The second experiment was performed with *A. brasilense* reisolated from associated callus cultures and maintained in SNF medium. Its growth and ARA were compared with the parent stock strain 7 and to strain JM125A2 (31). Each of 27 tubes containing 3 ml of SNF (0.05% agar) was inoculated with one 2-mm loopful of washed cell suspension (10^6 to 10^7 ml^{-1}) for each culture. Cultures were incubated at 35°C, sets of 9 being removed after 2, 3, and 4 days. The removed cultures were placed under 10% acetylene (see above), and three tubes each were incubated for 6, 12, and 24 h. Ethylene production was measured as above, and then the three tubes were pooled, centrifuged, and frozen at −20°C. Protein was determined on the pooled cells by the fluorescamine method.

Growth studies. These were performed by a stagnant culture technique (20) in replicate 40-ml vaccine vials containing 5 ml of medium (the high phosphate medium of Okon et al. [20], containing L-malate, 2 g $liter^{-1}$; KNO_3, 50 mg $liter^{-1}$ as starter N; and Ionagar no. 2, 0.05%, was used). The 0.05% agar produces an oxygen gradient in which the nitrogen-fixing *Azospirillum* isolates grow, at reduced oxygen tension, just below the surface of the medium. Inoculum was prepared from 24-h cultures (30°C) in Trypticase soy broth without dextrose (BBL Microbiology Systems) by washing twice, with centrifugation and resuspension in phosphate buffer (65 mM, pH 6.8). Cells were resuspended to give a density of ca. 0.8 optical density units, and 0.1 ml was added to each vial with mixing. Vials were incubated statically in a water bath at 30°C. At each of several incubation intervals (see Results), three replicate vials were removed, cotton stoppers were replaced with serum stoppers, and 10% of the gas volume was replaced with acetylene. Great care was taken to avoid disturbing the zones of subsurface growth, since mixing caused cessation of ARA. After 1 h of incubation (30°C), the vials were shaken, and samples of gas were withdrawn and assayed for ethylene. Growth was followed by measuring the absorbance at 560 nm of each vial. After determining the pH, the vials were frozen at −20°C. Two of the three replicate frozen vials from each set were later thawed, pooled, and assayed for nitrogen by the micro-Kjeldahl method.

Electron microscopy. Plate cultures were exposed to fumes from a stock solution of osmium tetroxide (4% aqueous solution) for 5 min, after which they were gently flooded with half-strength Karnovsky (17) fixative at room temperature and buffered in 0.1 M sodium cacodylate (pH 7.3). After 1.5 h, the plates were rinsed in the buffer, and colonies were gently removed from plates with a spatula and placed in a solution of 2% osmium tetroxide in the same buffer. After 1.5 h, the postfixative was carefully removed, and colonies were rinsed thoroughly in distilled water and then embedded in 2% agar. Bacteria in N-free culture tubes were pelleted and resuspended in the same fixative used for plate cultures. After the postosmium rinse, the pellets were embedded in 2% agar and processed, as were the agar-embedded whole colonies. Dehydration in a graded ethanol-acetone series included 1.5 h in 75% ethanol containing 2% uranyl acetate. The tissue was embedded in Spurr (28) plastic. Sections were poststained in uranyl and lead salts (25) and viewed in a Hitachi HU-11E, HU-11C, or Jeolco JEM 100CX transmission electron microscope.

The PASH stain for polysaccharide was performed on thin sections essentially as recommended by Pickett-Heaps (23). Light gold sections were cut, picked up on 300-mesh nickel grids, and oxidized in 1% periodic acid for 10 min at room temperature. After washing, sections were stained at 60°C for 20 to 30 min with silver hexamine reagent (11) and then treated with 1% Kodak fixer for 0.5 to 1.0 min to remove nonspecific staining. Sections were stained for 1 min with lead citrate or viewed without poststaining. A fine-grained silver deposit was obtained over polysaccharide-containing regions. For freeze-fracture studies, selected colonies from the plates used for standard fixations were removed, after rinsing off the primary fixative, and soaked in 20% glycerol for 20 to 30 min. They were frozen in liquid Freon-22 and then in liquid nitrogen, and freeze-fractured in a Balzers BA 360M freeze-etch device (18).

RESULTS

ARA. Comparison of the ARA of bacteria isolated from callus tissue with the parent *A. brasilense* strain 7 and with strain 13t (Table 1) showed appreciably higher values for the callus reisolates than for either of the stock strains. Callus-associated bacteria before maintenance on artificial medium had three- to fivefold higher specific activity (nanomoles of C_2H_4 $hour^{-1}$ milligram of $protein^{-1}$) than stock strains. When callus-associated bacteria were isolated and cultured in 3-ml SNF tubes, similar comparisons with *A. brasilense* strains 7 and JM125A2 were obtained (Table 2). The specific ARA of the reisolate was three- to eightfold higher than that for the other two strains. Standard deviations demonstrate high ARA variability for a given isolate (Tables 1 and 2). This is probably due to difficulty in keeping the subsurface growth band intact during processing for the acetylene reduction assay. Disturbance of the band exposes the

TABLE 1. *Specific acetylene reduction activities of Azospirillum: sugarcane callus isolate compared to strains 7 and 13t*[a]

Culture	Mean nmol of C_2H_4 h^{-1} $(tube)^{-1}$	Mg of protein/ tube	C_2H_4 (nmol h^{-1} mg of $protein^{-1}$)
13t	12.79 (±2.78)[b]	0.0606[c]	211
7	8.61 (±2.05)	0.0727	118
Callus isolate	38.71 (±10.13)	0.0637	608

[a] Incubated 6 days in SNF culture tubes (0.05% agar), 35°C. Inoculum: stock cultures of strains 7 and 13t and cells washed from callus pieces.

[b] Average (and unbiased standard deviation) of 12 3-ml SNF culture tubes after 24 h under 10% acetylene in air.

[c] Determined with cells pooled by centrifugation from 12 SNF culture tubes, by the fluorescamine method (5).

TABLE 2. *Specific acetylene reduction activities of Azospirillum strains 7 and JM125A2 and callus isolate with standardized inocula*[a]

Culture	Mean nmol of C_2H_4 h^{-1} (avg of 3 tubes)	Protein (mg $tube^{-1}$)	C_2H_4 h^{-1} (nmol mg of $protein^{-1}$)
JM125A2	8.29 (±6.54)[b]	0.0544 (±0.0172)[c]	153
7	2.89 (±2.45)	0.0490 (±0.0165)	59
Callus isolate	37.39 (±5.42)	0.0797 (±0.0172)	469

[a] One 2-mm loopful of suspension (10^6 to 10^7 ml^{-1}) inoculated into each 3 ml of SNF agar (0.05% agar).

[b] Data are averages of three SNF culture tubes each incubated for 2, 3, and 4 days at 35°C, and exposed to 10% acetylene in air for 6, 12, and 24 h (standard deviation based on nine data points for C_2H_2 reduction). Hourly rates of C_2H_4 production did not vary consistently with incubation ages and hours exposed to C_2H_2.

[c] Determinations by fluorescamine method made on cells pooled by centrifugation from three replicate tubes.

oxygen-sensitive nitrogenase enzyme to suboptimal concentrations of oxygen. The bacteria isolated from callus, whether at the vibrioid or C-form stage, were pure and identical to strain 7 microscopically and in colony form and development.

Growth studies. Growth rate as followed by changes in optical density at 560 nm was very similar for the callus reisolate and *A. brasilense* strain 7 (Fig. 1). The much larger increase in fixed nitrogen recorded for the callus reisolate between 10 and 36 h (Fig. 1) compared with that of *A. brasilense* strain 7 correlates well with the higher nitrogenase activity (ARA) of the callus reisolate at 22 and 36 h of growth (Fig. 2). The fact that this higher nitrogenase activity is not reflected in a higher growth rate for the callus reisolate would indicate that some factor other than nitrogen, perhaps oxygen, is limiting growth. The callus reisolate showed a peak ARA (at 22 h) about threefold that of strain 7 (at 36 h). The alkalinity in broth cultures increased steadily over the 60-h incubation period, from pH 6.95 at 10 h to pH 7.8 at about 60 h. Two additional experiments gave similar results.

Ultrastructure. Ultrastructural studies of whole colonies fixed in vitro to allow quantitative assessment of bacterial growth forms failed to indicate that the enhanced ARA of the reisolate was based on structural differences as compared with stock cultures. The structural features described below are similar for the reisolate and stock cultures.

When grown on plates with SNF medium, i.e., when fixing nitrogen in the presence of atmospheric pO_2, the bacterium produced a capsule (Fig. 3), giving a cellular morphology referred to here as C-forms. Capsulation was heaviest in cells near the surface of the colonies. This was

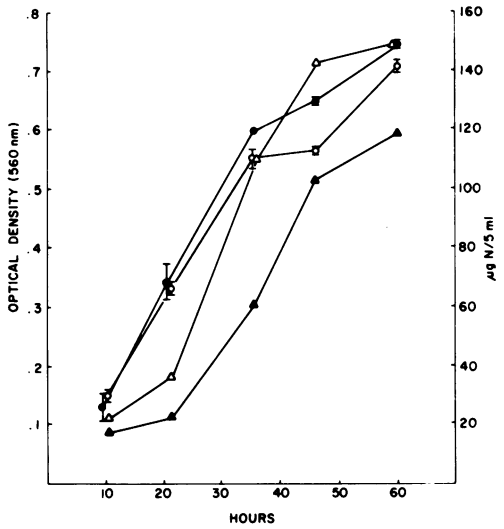


FIG. 1. *Growth of Azospirillum strain 7 and callus isolate in stagnant culture (20) containing 50 mg of "starter" KNO_3 per liter. Each point on optical density curve is average of three cultures; vertical bars represent standard deviation of the mean. Each value on N curve is one-half that determined on two pooled cultures. Symbols: ●, OD of strain 7; ○, OD of callus isolate; ▲, nitrogen content of strain 7; △, nitrogen content of callus isolate.*

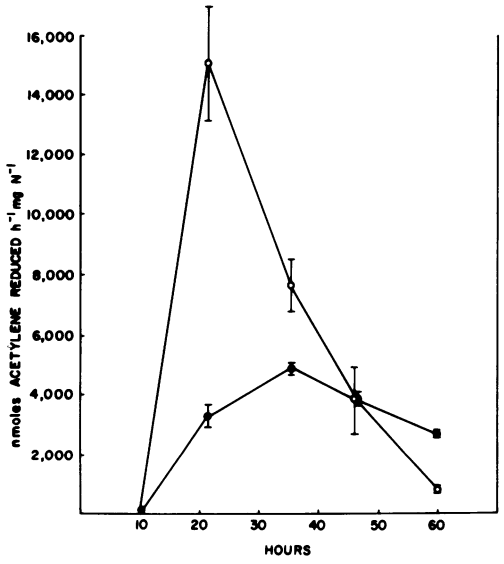
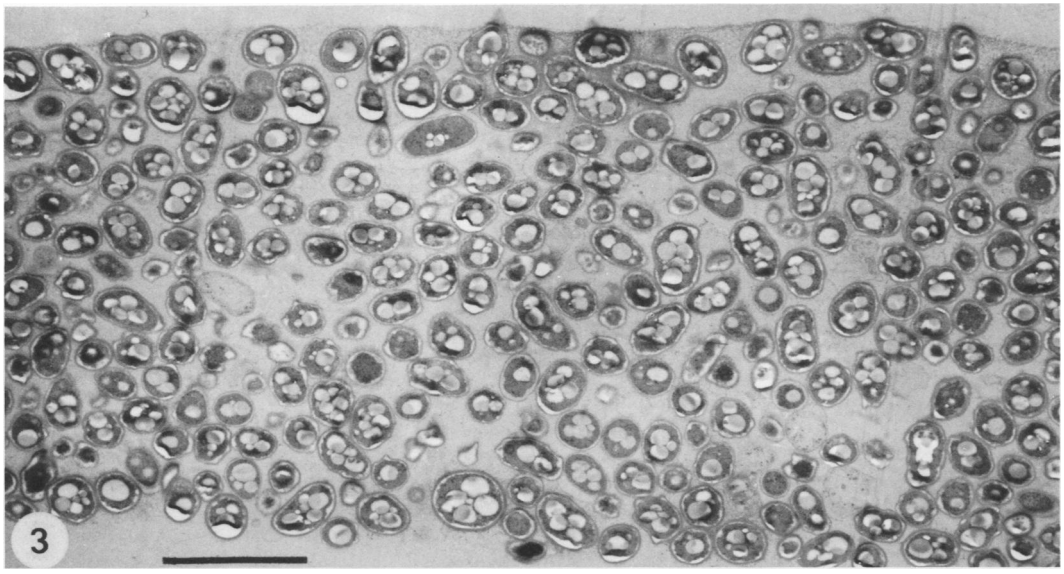
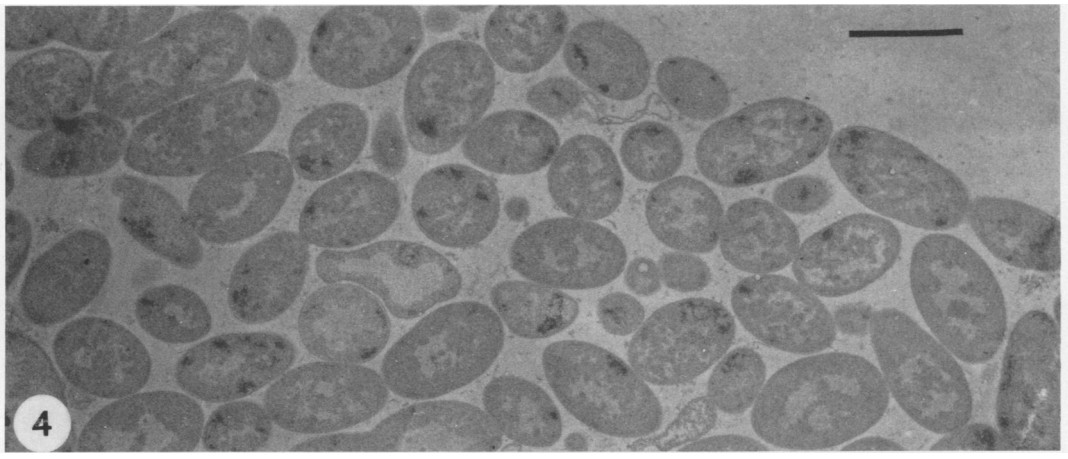
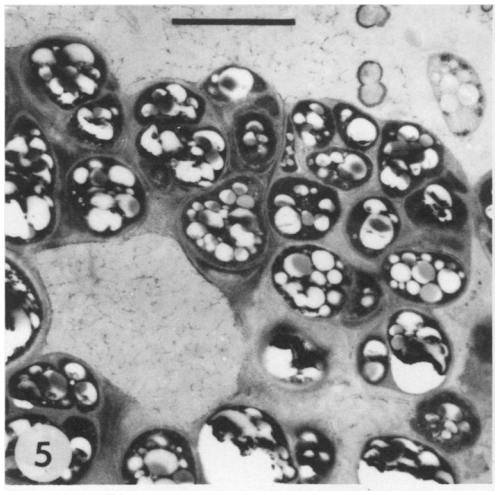


FIG. 2. *Acetylene reduction by Azospirillum strain 7 (●) and callus isolate (○) in stagnant culture (20) containing 50 mg of starter KNO_3 per liter. Each point is average of determinations on three cultures; vertical bars represent standard deviation of the mean.*

especially obvious as cultures on N-free media aged, and it resulted in a nearly continuous layer of C-forms across the surface of the colonies







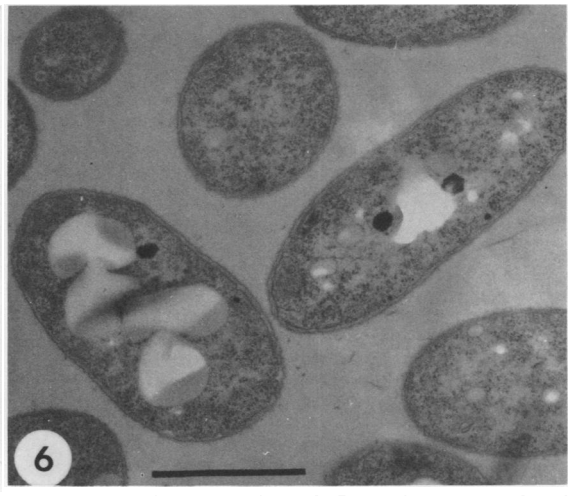


FIG. 3. *Thin section of Azospirillum strain 7 colony grown on a SNF plate for 48 h. Bacteria are capsulated (C-forms) and contain PHB. Marker represents 5 μm.*

FIG. 4. *Thin section of Azospirillum strain 7 colony grown on a plate containing SNF plus 0.25% NH_4Cl. Only a portion of this 48-h colony is shown. The bacteria are noncapsulated (V-forms) and lack PHB. Marker represents 2 μm.*

FIG. 5. *Thin section of heavily capsulated reisolate cells at the surface of an 8-day-old colony grown on a SNF plate. Marker represents 2 μm.*

FIG. 6. *Azospirillum strain 7 cells grown in SNF culture tubes for 60 h. No capsule is formed. Marker represents 1 μm.*

(Fig. 5). After this, cells in the interior of colonies also became more heavily capsulated. Size and shape changes in the C-forms are described later. Cells grown in culture tubes containing 3 ml of SNF did not produce a capsule during active growth (Fig. 6). The cells grew in a zone located below the surface of agar, presumably at the pO_2 optimal for growth under nitrogen-fixing conditions.

PASH staining for polysaccharides revealed the extensive nature of the capsular material (Fig. 7). The cell wall of *Azospirillum* is known to be gram negative. This was also true for C-forms (Fig. 8), where the capsule was shown to be external to the LPS layer. That the LPS layer was intimately associated with the capsule was evident in plasmolyzed cells where it remained tightly attached to the capsule (Fig. 9).

On SNF plates supplemented with combined nitrogen (0.25% NH_4Cl), rapid growth occurred of highly motile vibrioid cells which lacked a capsule ("V-forms," Fig. 4). The lack of capsulation was verified with PASH staining. The cytoplasm of C-forms contained large amounts of PHB, but cells grown on combined nitrogen appeared to have less (Fig. 4). The bacterium was found to be highly aerobic when grown on combined nitrogen, and smooth, round, white colonies developed rapidly. When fixing N_2, the bacterium was microaerophilic, and its growth was slow, resulting in smaller, irregular white colonies. Older colonies developed pink pigmentation.

The ability of C-forms of *Azospirillum* to fix N_2 has not been shown. This is because they were always found mixed with other forms of the bacterium and were difficult to separate. Also, they were most abundant in old cultures grown on N-free media where conditions such as pH were no longer conducive to nitrogen fixation. Thus, C-forms found in older (greater than 2 to 3 days) cultures may differ from simpler C-forms in respect to N_2 fixation.

When *A. brasilense* was grown on solidified SNF or other N-free medium, initial growth appeared as motile V-forms, approximately 1 by 3 μm in wet mounts. However, thin sections (Fig. 3) showed some of these to be capsulated (C-forms). As these cultures aged, C-forms appeared in a variety of shapes and sizes: from enlarged vibrioids (1.5 by 3.0 μm to 3 by 4.5 μm) to spheres (2- to 4-μm diameter). This was accompanied by a gradual loss of motility. Thin sections of the intermediary stages showed them to contain progressively fewer flagella and more cells per C-form, be it vibrioid or sphere. Some of the described forms are illustrated in Fig. 10 (wet mount). Refractile PHB granules were found in abundant quantity in pleomorphic forms (Fig. 10).

Spherical forms (C-forms) of a large diameter (5 to 8 μm) predominated in week-old cultures, and at this stage many pleomorphic cells could be found in each sphere. Heavy capsulation gave the C-forms a particularly adhesive nature, causing the formation of large, macroscopic clumps of cells (Fig. 11). Aging of cultures in N-free culture tubes was accompanied by the formation of large numbers of pleomorphic forms, some of which were capsulated.

Wet mounts of young *A. brasiliense* cultures grown on N-rich media showed virtually a pure culture of highly motile V-forms (general dimensions, 1 by 3 μm). As these cultures aged, combined nitrogen was depleted, the pH increased, and growth was slowed. At this stage most of the cells in the colony died. However, a small number of the advanced (i.e., large, multicelled, and spherical) pleomorphic forms were found in these cultures.

FIG. 7. *PASH staining of a reisolate C-form showing the extent of the capsule. Fine granularity represents silver deposited over polysaccharide-containing regions. Ten-minute periodic acid plus 30-min silver reagent. Tangential section. Marker represents 0.5 μm.*

FIG. 8. *Gram-negative cell wall structure of two adjacent reisolate C-forms. The cell membrane (white star) is obscured by peptidoglycan. The LPS (black star) has a nonstaining region on its outer surface. The capsule (C) is external to the LPS. Marker represents 0.1 μm.*

FIG. 9. *Degenerating reisolate C-form. The plasmolyzed cell membrane has separated from the LPS which remains attached to the capsule. Marker represents 0.1 μm.*

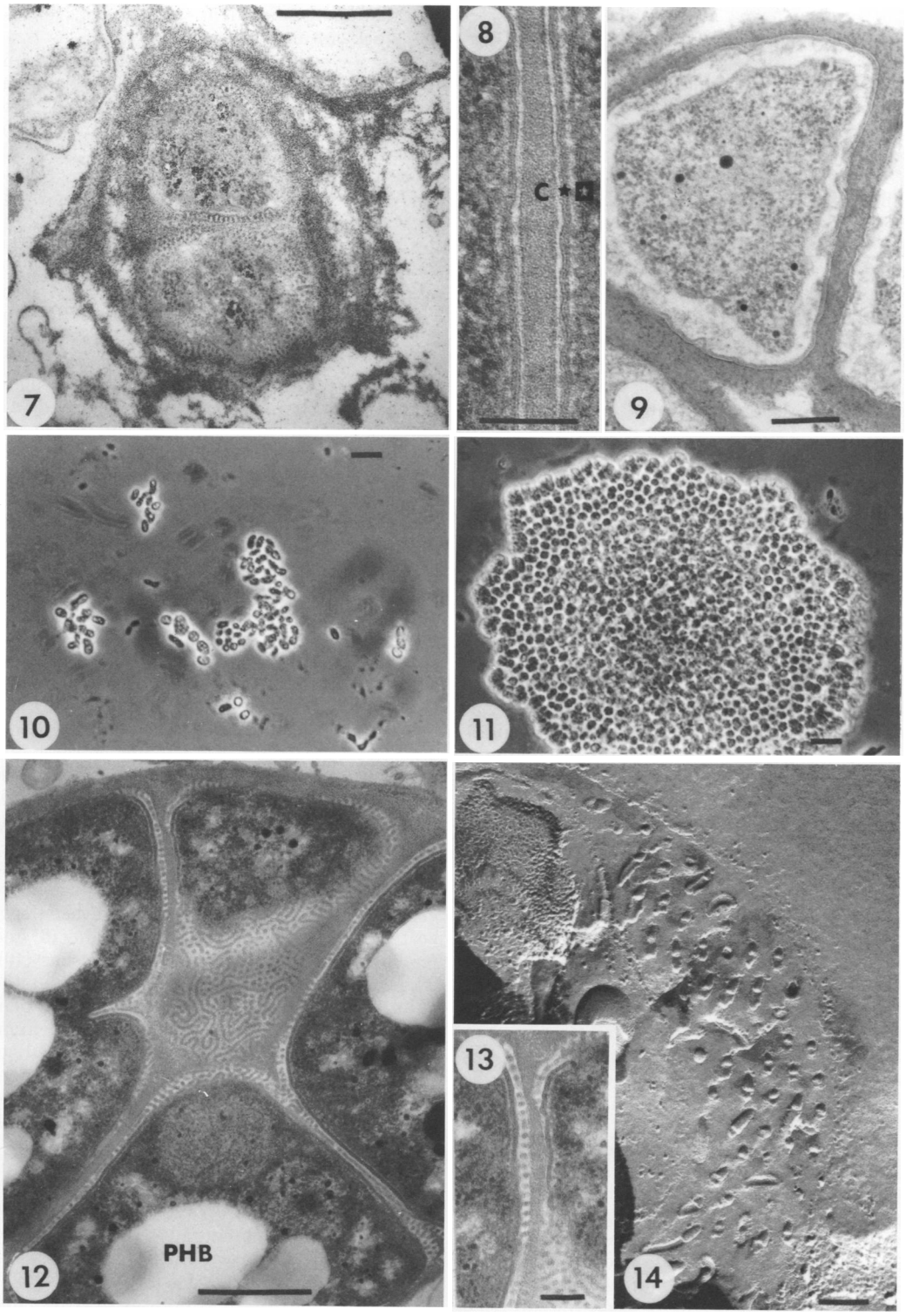
FIG. 10. *Wet mount of a 4-day-old Azospirillum strain 7 culture grown on a SNF plate. As cultures age C-forms become less motile and vary in size and shape as shown here. Oil immersion, bright field. Marker represents 6 μm.*

FIG. 11. *Wet mounts of culture of Fig. 10 at 7 days of age. At this stage, C-forms become large and rounded and contain several cells. Heavy capsulation causes these cells to adhere into large aggregates. Oil immersion, bright field. Marker represents 6 μm.*

FIG. 12. *The extracellular anastomosing tubules shown here (embedded in the capsule) were found in an occasional C-form. Marker represents 0.5 μm.*

FIG. 13. *Higher magnification of Fig. 12 showing the tubules to be extracellular to the intact LPS. Marker represents 0.1 μm.*

FIG. 14. *Freeze-fracture replica of tubules as found in Fig. 12. Marker represents 0.1 μm.*


7
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C
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In some C-forms an array of anastomosing tubules was found embedded in the capsule (Fig. 12 to 14). With the LPS in transverse section (Fig. 12 to 13) the tubules were in transverse section and were external to the intact LPS, indicating they were not a fixation artefact of the LPS. These tubules were never found clearly attached to the LPS. When the section was oblique to the LPS, the spatial distribution of the tubules was evident (Fig. 12). In this case, they appeared both in transverse and longitudinal section and also appeared to merge with the cytoplasm. The tubules stained similar to the LPS, which stained positive with the PASH stain (Fig. 7). With normal staining there was a nonstaining area immediately external to the LPS (Fig. 8, 13). The tubules also had a similar nonstaining region. The tubules are further illustrated in a freeze-fracture replica (Fig. 14).

DISCUSSION

The enhanced nitrogenase activity (ARA) of an *A. brasilense* strain cultured in association with sugarcane callus, although transient, may have material significance for energy conservation. Previous studies have shown only sporadic improvements in crop yield of forage grasses and cereals due to the presence of free-living N_2-fixing bacteria such as *Azotobacter paspali* (13) and *Azospirillum* (13, 26, 27). Furthermore, for both genera, phytohormones produced by the bacteria have been implicated as growth stimulants (2, 6, 30). Despite these cautionary findings, the proven, stable capacity of *Azospirillum* to fix nitrogen in pure culture and its wide occurrence in the rhizospheres of a variety of grass genera (15, 31) make it an attractive candidate for supplementing nitrogen nutrition in nonleguminous crops. The finding that *Azospirillum* can grow and persist on callus tissue cultures in N-free medium for many months offers an opening for research on potential improvement of bacterium-plant association. The elevated nitrogenase activity of the bacteria suggests a selection related to plant tissue effects. It remains to be shown that the bacterial persistence and enhanced activity seen in associated tissue cultures can be translated or manipulated to improve plant growth.

The ARA of strain 7 recorded herein lower than those noted by Okon et al. (22) and by Nelson and Knowles (19) may be attributed to differences in technique or changes during stock culture maintenance. Recent batch and continuous liquid cultures maintained under strictly controlled conditions in our laboratory have given results comparable to those of the cited workers. In any case, the significantly higher rates of ARA by the callus-associated strain were obtained under identical conditions for test and stock cultures.

It is not evident how callus association improved the ARA of *A. brasilense*. The bacterial activity involves a dilemma: O_2 is an absolute requirement as a terminal electron acceptor in providing adenosine triphosphate for both growth and for nitrogenase activity in N-free medium. At the same time, the nitrogenase is highly sensitive to inactivation by O_2. This genus, unlike *Azotobacter*, appears to have no innate anatomical protection of the enzyme against O_2. The sugarcane callus obviously provides adequate nutrition and oxidizable substrate for the bacterium. However, the bacteria grew exclusively in the intercellular spaces and on the surface of the callus, such that the plant tissue by itself did not evidently provide O_2 limitation. The formation of capsulated, bacteroid-like structures surrounded by a thick layer of carbohydrate-like polymer, here reported for the first time, suggests an adaptation which could control O_2 flow to the adenosine triphosphate-generating system and protect nitrogenase. Capsulated bacteria were rarely observed in liquid or deep agar cultures, and then only as aberrant forms in old cultures in N-free medium. However, C-forms were found to develop on surface-spread, N-free nutrient plates, whereas in tube cultures of N-free medium they occurred, if at all, only in very old cultures. Even mild disturbance of cultures in soft agar deeps destroyed ARA. In contrast, shaking cultures at a suitably low pO_2 enhanced ARA (21). These observations are consistent with the hypothesis that capsulation regulates O_2 flow to N-fixing cells.

It is conceivable that the callus may provide nutritional conditions which also enhance nitrogenase activity. Similar effects have been suggested for *Rhizobium* on soybean callus (3) and for *Azospirillum* on several callus tissues (10). The latter, however, occurred only when the culture medium was supplemented with a pentose. Our earlier work (4, 32, 33) has demonstrated prolific growth of *Azospirillum* and active ARA without pentose supplement. The enhanced ARA of callus-associated bacteria reported herein did not involve addition of pentoses to the medium.

Pleomorphic forms of *Azospirillum* have been previously reported (14, 16, 29; Tyler et al., Abstr. Int. Symp. N_2 Fixation, Brasilia, Brasil, p. 349–350, 1977). Generally, the forms have been described as helices, chains of vibrios, swollen rods, and medium to large ovoids, which developed in old cultures, notably in N-free medium. The suggestion that the ovoid forms may be equated with the cysts of *Azotobacter* and

related genera (16) was not confirmed by our study. The C-forms differ from *Azotobacter* cysts (24) morphogenically and morphologically. We have no evidence that the C-forms are resting or dormant forms of *Azospirillum*. Further elucidation of the genesis and the potential role of the C-form is being investigated.

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