Nitrogen Fixation Associated with Development and Localization of Mixed Populations of *Cellulomonas* sp. and *Azospirillum brasilense* Grown on Cellulose or Wheat Straw

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Mixed cultures of *Cellulomonas* sp. and *Azospirillum brasilense* were grown with straw or cellulose as the carbon source under conditions favoring the fixation of atmospheric nitrogen. Rapid increases in cell numbers, up to 10^9 cells per g of substrate, were evident after 4 and 5 days of incubation at 30°C for cellulose and straw, respectively. Nitrogen fixation (detected by acetylene reduction measured on parallel cultures) commenced after 2 and 4 days of incubation for straw and cellulose, respectively, and continued for the duration of the experiment. Pure cultures of *Cellulomonas* sp. showed an increase in cell numbers, but CO₂ production was low, and acetylene reduction was not detected on either cellulose or straw. Pure cultures of *A. brasilense* on cellulose showed an initial increase in cell numbers (10^7 cells per g of substrate) over 4 days, followed by a decline presumably caused by the exhaustion of available carbon substrate. On straw, *A. brasilense* increased to 10^9 cells per g of substrate over 5 days and then declined slowly; this growth was accompanied by acetylene reduction. Scanning electron micrographs of straw incubated with a mixed culture under the above conditions for 8 days showed cells of both species in close proximity to each other. Evidence was furnished that the close spatial relationship of cells from the two species facilitated the mutually beneficial association between them and thus increased the efficiency with which the products of straw breakdown were used for nitrogen fixation.

The concept of utilizing energy released by the microbial breakdown of straw to fuel the fixation of atmospheric nitrogen has been considered a viable means of increasing soil nitrogen and improving soil texture (8-10, 17). Various combinations of microorganisms have been proposed as suitable agents, capable of achieving this aim in either soil or compost. However, in systems which require an aerobic fungus as the cellulytic agent, the requirement for microaerobic or anaerobic conditions for N₂ fixation necessitates a separation in time or space of the various partners in the process (13, 17). This would lead to a partial loss of the products of cellulytic breakdown to other microorganisms in the soil. The use of anaerobic organisms for both cellulyolysis and N₂ fixation would require an anaerobic environment (e.g., waterlogged soil) and could result in the accumulation of phytotoxic products such as acetic acid (12).

The use of organisms capable of microaerobic growth for both cellulytic breakdown and N₂ fixation should increase the efficiency of the process. Increased efficiency has been demonstrated for mixed cultures of *Cellulomonas* spp. and *Azospirillum brasilense* (6, 7). In this system, the cellulytic activity of *Cellulomonas* sp. produces the substrate required by *A. brasilense* as an energy source for nitrogen fixation. The increased efficiency observed may arise from a close spatial relationship between these organisms, facilitating the utilization of the products of cellulytic breakdown, or from the low concentrations of this substrate available to the diazotroph, which could be expected to increase efficiency (3), or from both.

In an attempt to demonstrate the physical association between cells of the two species, single and mixed cultures of *Cellulomonas* sp. and *A. brasilense* were grown on straw and on pure cellulose under conditions requiring nitrogen fixation for growth. The increase in bacterial populations was monitored, and scanning electron microscopy was used to study the associations between cells of both species in mixed culture.

Controls consisting of pure cultures of *Cellulomonas* sp. and *A. brasilense* were included to determine the amount of growth which occurred in the absence of available nitrogen and carbon, respectively. Since *A. brasilense* can utilize the hemicellulose component of straw (6), growth could be expected over a short period in single culture.

**MATERIALS AND METHODS**

**Strains.** *Cellulomonas* sp. strain CS1-17, provided by N. Dunn, University of New South Wales, Kensington, New South Wales, Australia, is a mutant strain selected for its increased production of cellulase and reduced sensitivity to inhibition or repression by cellobiose and glucose accumulated in the culture medium (4, 5). *A. brasilense* Sp7 (ATCC 29145) (from J. Döbereiner, EMBRAPA, Sérropédica, Rio de Janeiro, Brazil) was provided by courtesy of F. J. Bergersen.

**Measurement of population increase in single and mixed cultures of *Cellulomonas* sp. and *A. brasilense*.** Increases in viable populations of *Cellulomonas* sp. and *A. brasilense* grown singly and as mixed cultures which were dependent on N₂ fixation for continued growth were monitored by using both cellulose and straw as carbon sources. The experimental design was factorial with 6 replicates of treatments in which each strain was grown separately and as a mixed culture on either straw or cellulose. Three replicates were used for plate counts, and three were used for estimation of C₂H₂ reduction and CO₂ production at 1, 2, 3, 4, 5, 8, 12, and 16 days after inoculation.

Folded strips of filter paper (1 g of Whatman no. 1) and wheat straw (1 g, cut into 50-mm lengths) were placed into
Universal bottles and autoclaved twice on successive days. The low-nitrogen medium used was based on the NFb medium of Döbereiner (2) and contained the following (per liter): MgSO₄·7H₂O, 0.2 g; NaCl, 0.1 g; CaCl₂, 0.02 g; Na₂MoO₄·2H₂O, 0.001 g; MnSO₄·H₂O, 0.01 g; iron-sodium-EDTA (1.64% wt/vol aqueous), 4.0 ml. The medium was buffered after autoclaving by the addition of sterile KH₂PO₄·NaOH (pH 7.0; 0.1 M final concentration), and filter-sterilized vitamins (biotin, 5 μg; thiamine, 10 μg; p-aminobenzoic acid, 10 μg) were added. No carbon source, other than the straw or cellulose, was added; however, a low level of nitrogen [2 μg/ml as (NH₄)₂SO₄] was added. Inocula of Cellulomonas sp. and A. brasilense grown and prepared as detailed in Halsall and Gibson (6) were introduced into flasks of low-nitrogen medium as both single and mixed cultures to give 4.5 × 10⁵ Cellulomonas cells per ml, 2.3 × 10⁵ A. brasilense cells per ml, or both. The inoculated medium (15 ml per bottle) was added to the cellulose or straw in Universal bottles which were loosely capped. Bottles were incubated at 30°C and were not shaken.

On each day of analysis, 3 replicates of each treatment were closed with sterile Suba seals (Wm. Freeman, Barnsley, United Kingdom) and C₂H₂ (3 ml) was introduced. The bottles were incubated for a further 24 h at 30°C, when the concentrations of CO₂, C₂H₄, and C₂H₂ in the gas phase (12 ml) were determined (6, 16). Another three replicates of each treatment were used to prepare three dilution series in 1/4-strength Ringer solution (1) with 0.01% Tween 20 to aid in the dispersion of the bacteria. The filter paper or straw pieces were removed from the culture medium with sterile forceps, drained, and placed in tube 1 of the dilution series.

After vigorous agitation for three periods of 1 min on a Vortex mixer (Vortex-Genie; Scientific Industries Inc., Springfield, Mass.) to suspend organisms closely associated with the substrate, each dilution series was completed, and 1 ml of the appropriate dilution was added to 2 ml of a soft agar overlay (1% water agar containing 0.1 mg of Congo red per ml) at 60°C and poured as a thin even layer over the surface of plates of potato infusion medium (2). Three plates were poured for each of three dilutions for Each replicate; colonies were counted after 5 days of incubation at 30°C. Congo red was taken up by A. brasilense colonies, which became bright pink (6), but it did not affect the growth or color of Cellulomonas colonies. The weight of cellulose or straw utilized was determined gravimetrically.

Electron microscopy. Cultures were grown under the conditions described above, and samples were taken for scanning electron microscopy after 8 days of incubation at 30°C. Straw and cellulose samples were fixed in 0.3% glutaraldehyde in phosphate-buffered saline (150 mM sodium phosphate buffer [pH 7.2], 0.85% NaCl) for 1 h at 20°C. After they were washed in phosphate-buffered saline, the samples were dehydrated in ethanol and then transferred to 100% dry acetone before critical-point drying by using CO₂ (A. G. Lane, personal communication). Dried material was placed on specimen stubs with double-sided adhesive tape, sputter-coated with gold, and examined in a JEOL 100 CX electron microscope fitted with an ASID4D SEM-STEM facility. Samples were examined and photographed at 20 kV in the scanning electron microscope mode.

RESULTS

Population growth in pure and mixed cultures of Cellulomonas sp. and A. brasilense grown on cellulose or straw. Mixed cultures of both Cellulomonas sp. and A. brasilense on cellulose, under conditions which favored the fixation of atmospheric nitrogen, grew rapidly over the first 4 days, from approximately 10⁶ cells per g of substrate to 3 × 10⁸ Cellulomonas cells per g of substrate and 1 × 10⁷ A. brasilense cells per g of substrate (Fig. 1a). From day 4 to day 16, viable numbers of Cellulomonas sp. remained relatively constant, whereas the numbers of A. brasilense increased gradually until day 12. From day 8 to day 16, a steady ratio of 1 A. brasilense cell to 4 to 5 Cellulomonas cells was maintained (Table 1). The mean weight of cellulose utilized over the 16-day period was 1.6 mg (1.6%).

FIG. 1. Increase over a 16-day period in bacterial populations of pure and mixed cultures of Cellulomonas sp. strain CS1-17 and A. brasilense Sp7 grown in unshaken low-nitrogen liquid culture (15 ml per bottle) with cellulose (a) or straw (b) as a carbon source under conditions favoring nitrogen fixation. Pure cultures of Cellulomonas sp. strain CS1-17 (△) and of A. brasilense Sp7 (○) and mixed cultures of Cellulomonas sp. strain CS1-17 (●) and A. brasilense (●) were examined.
Pure cultures of *A. brasilense* grown on cellulose increased in numbers over the first 4 days (Fig. 1a) but then declined, presumably because of the exhaustion of the small amounts of available carbon compounds in filter paper which could be degraded by this bacterium. The decrease in weight of the cellulose over the 16-day period was 0.1%. In contrast, pure cultures of *Cellulomonas* sp. were not apparently limited by a shortage of combined nitrogen, since cell numbers were maintained at 8 × 10^9 from day 4 until day 16. The estimate of total nitrogen in the filter paper was 76 µg of N per g (0.007%; G. L. Turner, personal communication), some of which may have been available for the growth of *Cellulomonas* cells. The mean weight of cellulose utilized was 1.6 mg (1.6%).

On straw, the mixed cultures grew rapidly, but the numbers of *Cellulomonas* were slightly below those obtained on cellulose over a 4-day period (1 × 10^9 cells per g of substrate) (Fig. 1b). *A. brasilense* grew better on straw than it did on cellulose (8 × 10^9 cells per g of substrate), but numbers declined slightly between 4 and 16 days. The ratio of *A. brasilense* to *Cellulomonas* sp. in mixed cultures grown on straw changed continuously, reaching 1 *A. brasilense* cell to 0.77 *Cellulomonas* cells on day 16 (Table 1). There was a 4.1% decrease in the weight of straw recovered after 16 days.

Pure cultures of *A. brasilense* on straw grew as rapidly as it did in the mixed culture, and viable cell numbers declined only slightly between 4 and 16 days (9 × 10^9 to 2 × 10^9 cells per g of substrate). Pure cultures of *Cellulomonas* sp. grew rapidly on straw to reach 6 × 10^9 cells per g of substrate on day 4 and maintained those numbers until day 16. The decrease in the weight of straw recovered after 16 days was 3.2% for *A. brasilense* and 3.5% for *Cellulomonas* grown in pure cultures.

Acetylene reduction and substrate degradation in pure and mixed cultures of *A. brasilense* and *Cellulomonas* sp. With cellulose as a substrate, nitrogenase activity (C_2H_2 reduction) was detected only in mixed cultures from day 4, when cell numbers were >10^9 cells per g of substrate, and reached a peak on day 12 (Fig. 2a). Degradation of the substrate, assessed by CO_2 production, was evident in all cultures, although it was consistently higher in the mixed culture. In pure cultures of both species, the level of CO_2 production showed an initial increase (from day 2 to day 4) and then decreased, whereas in the mixed culture, CO_2 production increased steadily over the 16-day period (Fig. 2c). Inoculated controls (established to determine CO_2 production from endogenous substrates [i.e., no C, no N controls]) produced less than 0.2 µmol CO_2 over 5 days.

With straw as the substrate, C_2H_2 reduction occurred from day 2 in the mixed culture and in pure cultures of *A. brasilense*. There was no evidence of C_2H_2 reduction by pure cultures of *Cellulomonas*. Nitrogenase activity in the mixed culture was greater than in the *A. brasilense* pure culture and was maintained over the 16 days of the experiment (Fig. 2b). A similar pattern of activity was evident for CO_2 production (Fig. 2d).

Electron microscopy of single and mixed cultures of *Cellulomonas* sp. and *A. brasilense*. Scanning electron micrographs were taken of cellulose fibers incubated with *Cellulomonas* sp. for 8 days (Fig. 3). The individual bacteria were variable in size, presumably because of differing growth stages, and varied in shape from short to long rods. This is consistent with the description (11) of 'irregular rods ca. 0.5 µm in diameter by 0.7-2.0 µm or more in length, which may be straight, angular or slightly curved and occa-

sionally club-shaped or beaded.' Samples of sterilized wheat straw incubated with *Cellulomonas* sp. contained bacteria with a similar morphology to those grown on cellulose.

Samples of straw incubated with *A. brasilense* for 8 days (Fig. 4) showed bacteria as described in Tarrand et al. (15); namely, 'short plump slightly curved rods averaging 1.0 µm in diameter and having a length of 2.1-3.8 µm.'

Scanning electron micrographs of straw incubated for 8 days with a mixed culture of these two organisms under conditions requiring straw breakdown and associated nitrogen fixation for cell growth showed many small aggregates in which the shorter narrower rods of *Cellulomonas* sp. and the larger plumper *A. brasilense* cells could be clearly distinguished (Fig. 5). The natural morphological variability observed in the two species resulted in an overlap in size and shape so that some cells could not be assigned categorically to either species. However, there were sufficient numbers of morphologically distinctive cells to demonstrate that aggregates did occur between cells from both species.

Since the cell aggregates were not distributed uniformly, and it was not possible to determine relative numbers of each species within an aggregate, estimates of cell numbers per unit of straw surface area could not be determined.

**DISCUSSION**

Mixed cultures of *Cellulomonas* sp. and *A. brasilense* were capable of rapid growth, and populations were sustained with either cellulose or straw as a carbon source and with atmospheric nitrogen as the principal nitrogen source. Populations built up rapidly to between 10^8 and 10^9 cells per g of substrate during the initial 3 to 4 days; subsequent changes were more gradual. A period of adjustment was necessary before a stable ratio of *A. brasilense* to *Cellulomonas* sp. cells was established between the two populations. On cellulose substrate, a stable ratio of 1 *A. brasilense* cell to 4 to 5 *Cellulomonas* cells was established in 8 days. On straw, a stable ratio did not develop within the 16-day duration of the experiment, probably because the ratio was affected by the presence of more readily degraded compounds such as hemicelluloses and soluble components which were available to *Azospirillum* (8) as well as to *Cellulomonas*. It has been established previously that these two organisms can sustain growth on cellulose and straw for periods of 30 to 40 days and that they fix atmospheric nitrogen during this period (7).

Pure cultures of *A. brasilense* could not maintain large viable populations when grown on cellulose, whereas on

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**TABLE 1. Ratio of *A. brasilense* Sp? to *Cellulomonas* Sp. strain CS1-17 cells grown in mixed culture on either cellulose or straw over a period of 16 days**

<table>
<thead>
<tr>
<th>Day</th>
<th>Cellulose</th>
<th>Straw</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>1:1</td>
<td>1:2</td>
</tr>
<tr>
<td>2</td>
<td>1:0.3</td>
<td>1:0.01</td>
</tr>
<tr>
<td>3</td>
<td>1:2</td>
<td>1:0.07</td>
</tr>
<tr>
<td>4</td>
<td>1:19</td>
<td>1:0.15</td>
</tr>
<tr>
<td>5</td>
<td>1:16</td>
<td>1:0.15</td>
</tr>
<tr>
<td>6</td>
<td>1:5</td>
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<tr>
<td>8</td>
<td>1:4</td>
<td>1:0.45</td>
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<tr>
<td>12</td>
<td>1:5</td>
<td>1:0.77</td>
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<tr>
<td>16</td>
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straw, large populations were maintained until the hemicellulose components of the straw were exhausted (8). Pure cultures of Cellulomonas sp. increased to 10^9 cells per g of substrate on both cellulose and straw in the absence of added nitrogen (other than the 2 μg per ml of medium), but respiration was low after day 4 (Fig. 2c), and there was no evidence of nitrogenase activity (Fig. 2a). This suggested either that Cellulomonas cells could scavenge NH_3 from the air or that the filter paper contained sufficient nitrogen to support growth on this scale (~0.01% N). Kjeldahl digestion and analysis of filter paper samples for nitrogen showed 75.7 μg of N per g (0.007%). If available, this in addition to the 30 μg of N present in the 15 ml of medium would have provided sufficient nitrogen for growth.

FIG. 2. Acetylene reduction (a and b) and CO_2 production (c and d) over a 16-day period by pure and mixed cultures of Cellulomonas sp. strain CS1-17 and A. brasilense Sp7 grown in unshaken low-nitrogen liquid culture (15 ml per bottle) with cellulose (a and c) or straw (b and d) as a carbon source under conditions favoring nitrogen fixation. Pure cultures of Cellulomonas sp. strain CS1-17 (A) and of A. brasilense Sp7 (○) and mixed cultures of Cellulomonas sp. and A. brasilense (●) were examined.

FIG. 3. Scanning electron micrograph of bacterial cells from a pure culture of Cellulomonas sp. strain CS1-17 grown for 8 days on cellulose filter paper in unshaken liquid culture with (NH_4)_2SO_4 as a nitrogen source. The cells were rod-shaped to slightly flexuous and varied in size. Bar = 5 μm.

FIG. 4. Scanning electron micrograph of bacterial cells from a pure culture of A. brasilense Sp7 grown for 8 days in unshaken low-nitrogen liquid culture with straw as a carbon source under conditions favoring nitrogen fixation. The cells varied in shape from short thin rods to plump rods, with some almost spherical. Bar = 5 μm.
FIG. 5. Scanning electron micrograph of cells from a mixed culture of *Cellulomonas* sp. strain CS1-17 and *A. brasilense* Sp7 grown for 8 days in an unshaken low-nitrogen liquid culture with straw as a carbon source under conditions favoring nitrogen fixation. The cells varied in shape from thin rods to plump rods, with some flexuous forms. Arrows, Aggregates of *A. brasilense* and *Cellulomonas* sp. cells in close proximity. Bar = 5 μm.

sufficient nitrogen to support the populations observed. The straw contained 7.09 mg of N per g (0.7%).

Scanning electron micrographs of straw samples taken 8 days after inoculation with a mixed culture of *Cellulomonas* sp. and *A. brasilense*, when cell numbers were relatively high and the cultures were actively fixing nitrogen, showed small aggregates of bacteria of both species in close proximity to each other. The procedure relies upon natural bacterial cell adhesion to the straw that may be stabilized by the fixative glutaraldehyde. Despite the possible loss of bacteria during the preparative procedures for scanning electron microscopy, there were many areas in which both bacterial cell types were observed. However, because of the preparative limitations and the topography of the specimens, it was not possible to assess the degree of coexistence in quantitative terms.

When *A. brasilense* and *Cellulomonas* sp. were grown together on straw or cellulose, very high levels of efficiency of nitrogen fixation were observed (72.4 mg of N per g of straw utilized) (7). In part, this high level of efficiency was due to the low concentration of substrate available to *A. brasilense* (3). However, the ability of the two organisms to coexist in the same microhabitat, as shown in this paper, is also an important factor in determining high efficiency. The low PO₂ conditions in these cultures are suitable for N₂ fixation by *A. brasilense*; they are also suitable for the growth of *Cellulomonas* sp., which differs from *Cellulomonas gelida* in growth and activity under these conditions (7). Without this close association, the products deriving from cellulase activity by *Cellulomonas* sp. would be dispersed and available to other soil bacteria and fungi. However, when a close association exists, product transfer is more direct and losses due to the activity of other soil organisms will be minimized.

In Australia, wheat stubble is either burned or plowed into the soil. The latter practice has been shown to control erosion and improve soil structure (14). In two locations where stubble incorporation has been practiced for 10 to 12 years, high levels of nitrogenase activity in the soil have been demonstrated (14). Roper suggests that in soils in which straw retention practices have been adopted, there is a considerable potential for nitrogen fixation. One possible method of facilitating straw breakdown and the associated nitrogen fixation could involve the use of a mixed inoculum of cellulosytic and diazotrophic organisms. Field studies using these two organisms as an inoculum are currently in progress.

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