

## An Asymbiotic Nitrogen-Fixing Bacterium from the Root Environment of Corn

(*Enterobacter cloacae*/acetylene reduction/nonleguminous plants)

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**ABSTRACT** Examination of root systems and adhering soil associated with unusually green corn plants from nitrogen-deficient areas in fields has revealed higher acetylene-reducing activities than comparable root systems of chlorotic plants that appeared to be nitrogen deficient. From the root systems with acetylene-reducing activity, *N*<sub>2</sub>-fixing *Enterobacter cloacae* were isolated. Pure cultures of the organism grown in a medium lacking added fixed nitrogen reduced acetylene to ethylene and used *N*<sub>2</sub> as a sole source of nitrogen under anaerobic conditions. Acetylene reduction was strikingly inhibited by 0.04 atm or greater partial pressures of *O*<sub>2</sub>; however, the bacteria maintained appreciable acetylene-reducing rates in medium exposed to partial pressures of *O*<sub>2</sub> ranging from 0.005 to 0.015 atm. Nitrogenase activity in cell-free extracts of the bacterium was dependent upon *Na*<sub>2</sub>*S*<sub>2</sub>*O*<sub>4</sub> and an ATP-generating system. Some environmental conditions expected to influence *N*<sub>2</sub> fixation by free-living *N*<sub>2</sub>-fixing bacteria on root surfaces of nonlegumes are discussed.

The occurrence of asymbiotic, *N*<sub>2</sub>-fixing bacteria in soils has been reported by many investigators (1-3). Quastel (4) discussed microbial activity in the rhizosphere of many cultivated plant species but presented no evidence for concentration of *N*<sub>2</sub>-fixing bacteria in the rhizosphere. An increase in total nitrogen content of soil by growing rye grass was reported by Parker (5), who concluded that *N*<sub>2</sub> may be fixed in the rhizosphere by microorganisms using root excretions. According to Willis and Green (6), sufficient *N*<sub>2</sub> was fixed to compensate for nitrogen use by rice plants when they were grown under flooded conditions. They suggested that free-living *N*<sub>2</sub>-fixing bacteria may be responsible for the fixation. Nitrogen-fixing *Klebsiellae* have been identified (7) consistently on the surfaces of roots and nodules of soybeans, alfalfa, and clover. Associations of *Azotobacter paspali* with roots of *Paspalum notatum* and *Beijerinckia indica* with roots of sugar cane have been observed in tropical soils by Döbereiner (8). There is relatively little detailed information, however, concerning *N*<sub>2</sub> fixation in the root environment of nonleguminous plants. This communication reports the occurrence of *N*<sub>2</sub>-fixing bacteria in the root environment of corn plants (*Zea mays*, Pioneer 3773) and describes the *N*<sub>2</sub>-fixing capability of a representative isolate identified as *Enterobacter cloacae*.

### MATERIALS AND METHODS

*Media.* For isolation of *N*<sub>2</sub>-fixing bacteria from the root environments and for subsequent experiments, Hino and

Wilson's (9) medium (H and W medium) containing 50 ppm *CaCl*<sub>2</sub>, instead of *CaCO*<sub>3</sub>, was used. The bacteria were maintained on slants of H and W medium containing agar, 30 ppm of bromothymol blue, and 10 g/liter of *CaCO*<sub>3</sub>, instead of *CaCl*<sub>2</sub>. Vitamin-free casamino acids (DIFCO) and nodule extract were used to supplement the H and W medium in studies on the effect of combined nitrogen on acetylene reduction by the bacterium. Casamino acids were added to the medium before it was autoclaved, while nodule extract (7) was sterilized by filtration and then added to the sterile medium.

*Isolation of Bacteria.* Modified Pankhurst tubes ("H" tubes) were used in isolation of the facultatively anaerobic bacteria (10, 12). Part A of H and W medium (9.8 ml) was added to one of the arms of each "H" tube before it was closed loosely with a screw cap. The other arm was fitted with a sleeve-type serum stopper, and the assembly was sterilized by autoclaving. Part B of the medium (0.2 ml), sterilized separately, was added aseptically. Pieces of roots and adhering soil from the corn root systems exhibiting acetylene-reducing activity were added to the medium in one arm of each "H" tube before a serum stopper was inserted. Procedures for flushing with *N*<sub>2</sub>, maintenance of anaerobic conditions, introduction of acetylene, and sampling for ethylene have been described (10).

Serial dilutions of cultures in "H" tubes exhibiting positive nitrogenase activities were made, and aliquots were plated on H and W agar by the pour-plate procedure. The plates were incubated anaerobically at room temperature under *N*<sub>2</sub>. Representative colonies from the plates were used for inoculating more "H" tubes and were later tested for nitrogenase activity. Cultures possessing acetylene-reducing capacity were plated on H and W medium and incubated as before. The procedure was repeated four times to ensure purity of cultures.

*Diagnostic Biochemical Tests* were conducted at 37° by procedures recommended in the Manual of Clinical Microbiology (11). The techniques for extraction and purification of DNA and the procedure for determining the guanine plus cytosine base composition of DNA were reported earlier (12).

*Preparation of Cell-Free Extracts.* A 33-liter culture of the bacteria was grown for 48 hr in H and W medium that was flushed continuously with high-purity *N*<sub>2</sub>. The large culture was inoculated with a 48-hr-old culture (620 ml with an OD of 0.18) grown in H and W medium supplied with *N*<sub>2</sub>. The cells from large cultures were harvested by continuous flow centrifugation. Large quantities of capsular slime produced

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by the bacterium adversely affected collection of cells by centrifugation. The centrifuge had to be stopped at intervals, and the cells and thick slime were transferred to other tubes for further separation of cells. These transfers and other manipulations were done under a stream of N<sub>2</sub> or in a glove box filled with N<sub>2</sub>. The collected cells were pooled, suspended in 0.1 M TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) (pH 8.4) and centrifuged at 40,000 × *g* for 30 min. The sedimented bacteria with some slime were frozen in liquid N<sub>2</sub> and stored until used. In preliminary trials, 25 mM TES (pH 7.4) was used to resuspend the cells, but this concentration of buffer was insufficient to prevent a marked decrease in pH. In all subsequent trials, therefore, 0.1 M TES buffer at pH 8.4 was used to prevent increases in acidity.

For preparation of the crude extract containing nitrogenase, the bacterial cells in a minimum volume of buffer were allowed to thaw under N<sub>2</sub> and were disrupted under anaerobic conditions with an Aminco French Pressure cell at a pressure of 16,000 lb./inch<sup>2</sup>. The macerated cells were collected under N<sub>2</sub> in polycarbonate tubes containing 0.05 mg each of RNase and DNase, and centrifuged at 40,000 × *g* for 60 min. The supernatant (crude extract) was used immediately in the cell-free enzyme experiments.

*Ethylene Was Assayed* by use of a Varian-Aerograph model 600 D gas chromatograph equipped with a hydrogen flame detector and a column (6 ft. × 1/8 inch) of Poropak R and operated at a temperature of 50°.

*Turbidities of Cultures Were Measured* in a Bausch and Lomb Spectronic 20 at a wavelength of 540 nm. Turbidities of cultures in "H" tubes were determined directly without opening the cultures.

*Proteins.* The Biuret method was used for the determination of proteins in cell-free extracts (13).

## RESULTS

*Acetylene Reduction in the Root Environment of Corn.* The amount of acetylene reduced by two root systems of green corn ranged from 578 to 689 nmol in 42 hr (Table 1). Root systems of chlorotic corn plants exhibited relatively little acetylene-reducing activity, and soil samples collected between the rows of corn reduced less than 1–27 nmol in 42 hr. It was evident, therefore, that most of the acetylene-reducing activity was associated with the root systems of green corn plants.

*Isolation of Bacteria.* We attempted to culture organisms responsible for acetylene reduction by incubating pieces of corn roots and small quantities of rhizosphere soil aerobically in Burk's sucrose medium at 25°. Neither appreciable growth nor measurable acetylene reduction was observed during an incubation period of 2 weeks. Incubation of samples of roots and adhering soil from green corn plants was repeated, except that H and W medium and anaerobic conditions in "H" tubes were used. Visible growth and evolution of gas was observed in all tubes within 24 hr. Cultures (10 ml) incubated for 48 hr reduced acetylene at rates ranging up to 4794 nmol in 24 hr. There cultures exhibiting maximum activity were used for dilution plating. From plates incubated anaerobically, three isolates with high acetylene-reducing capabilities were obtained.

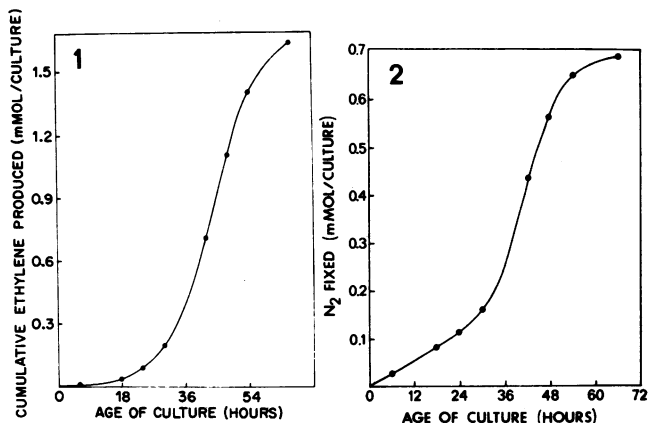


FIG. 1. Rate of acetylene reduction by *E. cloacae*. H and W broth (620 ml) was inoculated with 20 ml of an anaerobic culture (OD .18) maintained in "H" tubes and grown in a 1-liter aspirator bottle that was flushed continuously with sterile N<sub>2</sub>. A rubber hose (5 cm long) with a glass tube fitted with a sleeve-type serum stopper was attached to the outlet of the aspirator bottle. Aliquots (2 ml) of each growing culture were aseptically removed at 6-hr intervals by sterile syringes and were tested for acetylene-reducing activity in 21-ml bottles previously filled with O<sub>2</sub>-free argon. Acetylene (2 ml) was introduced into each bottle. The bottles were then incubated at 30° with shaking in a water bath for 1 hr. Gas samples (0.5 ml) were quickly removed for measurement of ethylene. Four replicate subcultures were removed at each age indicated, and mean C<sub>2</sub>H<sub>2</sub> reduction rates from these were used for calculation of the cumulative acetylene reduction.

FIG. 2. Rate of N<sub>2</sub> fixation by *E. cloacae*. The organism was cultured as described in the legend of Fig. 1. Samples (0.2 ml) were removed from the culture by a sterile syringe and analyzed for nitrogen by a modified microkjeldahl method. Results presented are means of four replicate samples, each corrected for the nitrogen content of the medium and the inoculum.

*Biochemical Characteristics of Isolates.* The following characteristics of the three different isolates (all apparently identical) were: Gram reaction, negative; growth on triple sugar iron agar (slant and butt), A (acid); H<sub>2</sub>S production, negative; indole production, negative; methyl red reaction, negative; Voges-Proskauer (acetoin production), positive; oxidase reaction, negative; urea hydrolysis, negative; arginine dihydrolase, positive; lysine decarboxylase, negative; ornithine decarboxylase, positive; motility, positive; gelatin hydrolysis, negative.

The following results were obtained from tests of the use of carbon sources: citrate, positive; lactose, AG (acid and gas); glucose, AG; manitol, AG; sucrose, AG; mannose, AG; maltose, AG; fructose, A; glycerol, negative; inositol, negative; cellibiose, positive; dulcitol, negative; sorbitol, positive; glycogen, negative; raffinose, positive; arabinose, positive; dextrin, positive; xylose, positive; rhaminose, positive; galactose, positive; inulin, positive. The DNA guanine and cytosine content of the isolates ranged between 52 and 53%. From the negative indole and methyl red tests, positive Voges-Proskauer test, ability to use citrate, and the typical triple sugar iron agar response, the cultures were conclusively identified as members of the *Klebsiellae* (11). The guanine and cytosine DNA base composition of 52–53% supports the phenotypic identification. Generic identification as *Enterobacter* was made on the basis of motility, the presence of flagella, and the results of decarboxylase reactions. The most significant fea-

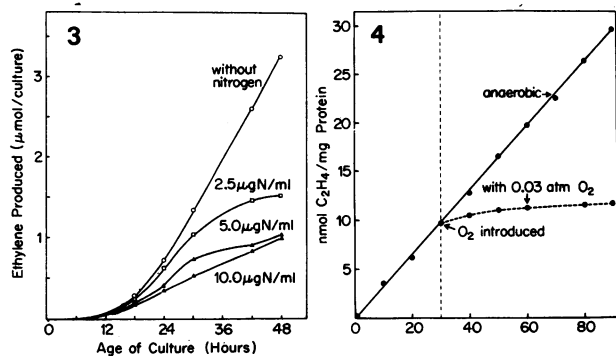


FIG. 3. The effect of added Casamino acids on cumulative acetylene reduction by *E. cloacae*. A series of 10-ml cultures in "H" tubes containing H and W medium were grown anaerobically with the different concentrations of added nitrogen in the form of Casamino acids. Three replicate cultures were removed and assayed for acetylene reduction after a 1-hr incubation period (see legend, Fig. 1), and the results were used for calculation of cumulative ethylene production.

FIG. 4. Oxygen inhibition of acetylene reduction by *E. cloacae*. The culture was grown, sampled, and assayed for acetylene reduction by the procedures described in the legend of Fig. 1. Oxygen (0.03 atm) was introduced into vessels after incubation with acetylene for 30 min. Four replicate samples were assayed at 10-min intervals, and mean values are presented. Protein content was calculated from the total nitrogen of each sample.

tures permitting species identification as *E. cloacae* include the presence of arginine dihydrolase and ornithine decarboxylase, and the inability to ferment inositol, dulcitol, and glycerol (11).

**Acetylene Reduction and Nitrogen Fixation.** All three isolates cultured anaerobically in a nitrogen-free medium grew after a lag period of 18 hr and rapidly reduced acetylene (Fig. 1). Rates of acetylene reduction decreased considerably after 54 hr. After a culture period of 42 hr the culture became viscous with slime, and turbidity measurements were no longer meaningful. The trend of  $N_2$  fixation by a comparable culture (Fig. 2) was similar to that showing cumulative acetylene reduction. The nitrogen in cells from a 10-ml culture (analogous to that described in Fig. 2) that had been exposed to 0.1 atm of 98%  $^{15}N_2$  for 48 hr contained 20.5 atom percent  $^{15}N$  excess over the normal abundance of  $^{15}N$ . This finding confirms the  $N_2$ -fixing capability of this organism.

Supplementation of the medium with either Casamino acids (Fig. 3) or root nodule extract (not presented) inhibited the acetylene-reducing activity of *E. cloacae*. Addition of 2.5  $\mu$ g of nitrogen per ml as Casamino acids reduced the rate of acetylene reduction more than 50%. Addition of higher amounts of fixed nitrogen as Casamino acids produced less striking effects.

**Inhibition of Acetylene-Reducing Activity by Oxygen.** Little or no growth of *E. cloacae* was observed in cultures incubated anaerobically in H and W medium with shaking. When 0.03 atm of  $O_2$  was introduced into an anaerobic culture, the acetylene-reducing activity practically ceased within 10 min (Fig. 4).

The effect of different partial pressures of  $O_2$  on the acetylene-reducing activity of samples removed from an anaerobic

$N_2$ -fixing culture are presented in Fig. 5. Activity was completely inhibited by addition of 0.04 atm of  $O_2$ .

**Acetylene Reduction by the Cell-Free System.** The data presented in Table 2 show that acetylene reduction in reaction mixtures containing a cell-free extract of *E. cloacae* was dependent upon the ATP-generating system and  $Na_2S_2O_4$ . Results from reactions in which individual components of the ATP-generating system were omitted establish an absolute requirement for ATP. The limited activity observed in the absence of added creatine phosphokinase indicates that the extract must have contained this enzyme. The low activity observed in the absence of added creatine phosphate and creatine phosphokinase may be accounted for by the 7.5  $\mu$ mol of ATP that was included in the assay. The very low activity recorded where no  $Na_2S_2O_4$  was added to the standard assay undoubtedly is due to the  $Na_2S_2O_4$  added to buffers during preparation of cell-free extracts. From this experiment (Table 2) it appears that the requirements for activity of *E. cloacae* nitrogenase are essentially the same as those of cell-free nitrogenase preparations of many other  $N_2$ -fixing organisms.

## DISCUSSION

The acetylene-reducing activity in the root environment of green corn plants from fields was substantially higher than that in either the root environments of chlorotic plants or in samples of soil taken between rows of corn (Table 1).  $N_2$ -fixing *E. cloacae* were consistently isolated from root systems that showed acetylene-reducing activity. These results are consistent with those of a previous study (7), which demonstrated a concentration of  $N_2$ -fixing *Klebsiellae* in the rhizosphere of certain leguminous species. From rates of acetylene reduction by root systems of green corn we have estimated that  $N_2$  fixation by organisms in the root environment contributes less than 0.5 kg of fixed nitrogen per hectare. This quantity is relatively insignificant when compared with the results of Döbereiner *et al.* (14), who estimated that *Azoto-*

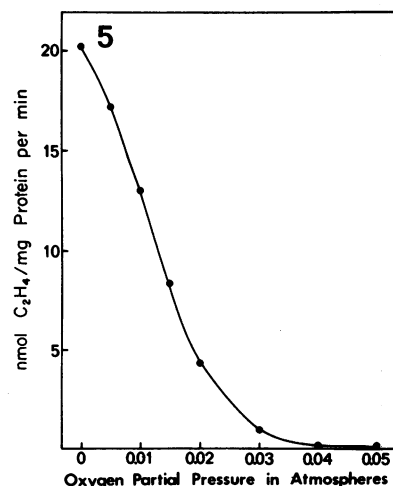


FIG. 5. Effect of different partial pressures of  $O_2$  on the rate of acetylene reduction by *E. cloacae*. The experimental procedure was the same as that described in the legend of Figs. 1 and 3, except that  $O_2$  at the partial pressures indicated was introduced into sample bottles in addition to acetylene. Rates of acetylene reduction were calculated from means of four replicate samples incubated for 1 hr.

*bacter paspali* on the roots of *Paspalum notatum* may fix as much as 90 kg/hectare.

Although we have demonstrated acetylene-reducing activity and the presence of a N<sub>2</sub>-fixing *Enterobacter cloacae* in the root environment of corn, we have not shown that this bacterium is involved in a biological association with corn roots in a manner analogous to the association of *Azotobacter paspali* with the roots of *Paspalum notatum* (14). An appropriate question is whether the apparent vigor of green corn showing acetylene-reducing activity around the roots is a consequence of N<sub>2</sub> fixation by *E. cloacae* or is the concentration of N<sub>2</sub>-fixing bacteria in the root environment a response to available carbohydrate supplied by the relatively vigorous plants? To answer this question, corn must be cultured in a sterile nitrogen-deficient medium with and without N<sub>2</sub>-fixing *E. cloacae*.

*E. cloacae* produced an enormous amount of slime when cultured in a nitrogen-free medium. The physiological role of bacterial slime as a possible means of limiting O<sub>2</sub> concentration in the vicinity of the O<sub>2</sub>-sensitive N<sub>2</sub>-fixing sites has been discussed by Postgate (15) and by Hill (16). Brown (17) has pointed out that the transfer of O<sub>2</sub> in bacterial cultures is decreased appreciably by slime, and Hill (16) has shown that massive colonies of *Derxia gummosa* that contained large quantities of slime reduced acetylene, whereas small colonies presumably containing less slime exhibited little or no acetylene-reducing activity. We have no evidence that *E. cloacae* on root surfaces of corn produce slime, but we have observed that some of the roots of wild rye, wheat, and triticale collected from fields in Oregon are coated with a mass of colloidal soil material that seems to be attached by root hairs and by material that may be bacterial slime. From roots of this type, N<sub>2</sub>-fixing bacteria have been consistently isolated. It seems reasonable to assume that the production of slime by bacteria on the root surfaces of grasses may be an important physiological adaptation favoring the growth of N<sub>2</sub>-fixing bacteria. Oxygen-sensitive N<sub>2</sub>-fixing organisms, such as *E. cloacae*, would be expected to require some type of mechanism to enable them to establish a partially anaerobic microenvironment favorable for N<sub>2</sub> fixation.

TABLE 1. Acetylene reduction in the root environment of corn plants

Sample no.	Material*	Acetylene reduced (nmol)	
		18 hr	42 hr
1	Root system from chlorotic corn	43	43
2	Root system from chlorotic corn	17	85
3	Root system from chlorotic corn	43	51
4	Root system from green corn	128	689
5	Root system from green corn	298	578
6	Soil about 18 inches from corn	—	27
7	Soil about 18 inches from corn	<1	<1

\* Mature corn plants from a nitrogen-deficient area in a field near the Oregon State University dairy farm were examined in September, 1971. Each sample consisted of the root system of an intact plant with about 2 kg of adhering soil or of a 2-kg sample of soil. Acetylene-reducing capacities were tested in 21-liter plastic containers to each of which was added 0.01 atm of C<sub>2</sub>H<sub>2</sub>.

TABLE 2. Acetylene reduction by cell-free extract of *E. cloacae*

Reaction	Specific activity (nmol C <sub>2</sub> H <sub>4</sub> /mg protein per min)
Complete*	5.0
Without ATP system†	0.0
Without creatine phosphate	0.5
Without creatine phosphokinase	1.3
Without ATP	0.0
Without Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub>	0.2
Without C <sub>2</sub> H <sub>2</sub>	0.0

\* The complete reaction in a final volume of 1.5 ml contained: 50 μmol creatine phosphate, 0.5 mg creatine phosphokinase, 7.5 μmol ATP, 10 μmol MgCl<sub>2</sub>, 100 μmol TES buffer (pH 7.5), and 20 μmol Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> in 0.05 M TES buffer (pH 7.4). All reagents except Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> were added, reaction vessels (21 ml volume) were flushed eight times with high-purity argon, and the Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> solution was injected into each bottle. *E. cloacae* extract (0.9/mg protein) was injected into each vessel, and reactions were initiated by injection of 2 ml of acetylene. After incubation with shaking for 30 min in a water bath at 30°, reactions were terminated by injection of 0.5 ml of 50% trichloroacetic acid. 0.5-ml gas samples were removed for measurement of ethylene.

† Consisted of Na<sub>2</sub>ATP, creatine phosphate, and creatine phosphokinase.

N<sub>2</sub>-fixing *Klebsiellae* have been described (1, 7, 12, 18, 19). Mahl *et al.* (18) reported that 13 of 61 strains of *Klebsiellae* were capable of fixing N<sub>2</sub> gas. Fixation was demonstrated by representatives of *K. pneumoniae*, but strains of *Serratia* and *Enterobacter* were negative. Recently the isolation of a culture of N<sub>2</sub>-fixing *Enterobacter* was described by Bergersen and Hipsley (19) and Line and Loutit (1). Bergersen and Hipsley tentatively identified one N<sub>2</sub>-fixing strain as *E. cloacae*, but the few traits examined by them make it difficult to differentiate *E. cloacae* from *E. aerogenes*. According to Line and Loutit (1), N<sub>2</sub>-fixing *K. pneumoniae* and *E. aerogenes* from soils in New Zealand have been isolated. If the identification of these organisms as *Klebsiellae* is accepted, we must consider the origin and significance of *E. cloacae* and *K. pneumoniae* in cultivated soils since these organisms are inhabitants of animal feces.

Ouellette *et al.* (20) report 57–63% guanine and cytosine in the DNA of *Klebsiellae*. These are the only values reported specifically for N<sub>2</sub>-fixing cultures. This range is significantly higher than the 52–56% guanine and cytosine content determined for *Klebsiellae* that do not fix N<sub>2</sub> by De Ley (21). The question then arises as to whether the N<sub>2</sub>-fixing strains are genetically unique. From our results and from an earlier study (12), it would seem some N<sub>2</sub>-fixing cultures have mean guanine and cytosine contents comparable to those of *Klebsiellae* that do not fix N<sub>2</sub>.

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