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CHARACTERISTICS OF NITROGEN-FIXING *KLEBSIELLA OXYTOCA* ISOLATED FROM WHEAT ROOTS

by M. LUTFU CAKMAKCI*, H. J. EVANS** and R. J. SEIDLER***

KEY WORDS

Acetylene reduction Inoculation *Klebsiella oxytoca* Nitrogen fixation Wheat

SUMMARY

Of 45 fermentative gram negative bacterial isolates examined from wheat roots, three were capable of fixing atmospheric nitrogen as determined by the acetylene reduction technique and by protein contents of cells. A gram negative non-motile facultatively anaerobic bacterial strain capable of N_2 fixation was identified as *Klebsiella oxytoca* ZMK-2.

Optimal growth and N_2 fixation occurred at pH 6.5. The optimum temperatures for growth under anaerobic conditions ranged between 30°–37°C. Acetylene reduction by intact cells was strikingly inhibited by 0.1 atm. or greater partial pressure of O_2. Furthermore, the accumulation of H_2 in the gas phase over cultures of *Klebsiella oxytoca* ZMK-2 at partial pressures greater than 0.02 atm. resulted in a striking inhibition in the rate of C_2H_2 reduction. The addition of suspensions of either *Klebsiella oxytoca* ZMK-2 or *Azotobacter vinelandii* or a mixed culture of these two organisms to axenic cultures of wheat plants produced no significant increase in plant growth as measured by plant dry weight or nitrogen content of plants.

INTRODUCTION

Biological nitrogen fixing capability is observed in different groups of microorganisms, including obligate anaerobes, obligate aerobes, facultative anaerobes and microaerophilic bacteria. In the last two decades, much work has been directed toward an understanding of the biology and ecological relationships of nitrogen fixing organisms. Considerable attention has been devoted to associative symbioses between free-living nitrogen fixing bacteria and roots of certain plants[3]. In an effort to better understand the mutual metabolic interactions between plant roots and microorganisms, composition of root exudates were determined and their effects on interactions between plants and bacteria tested[22]. Nitrogen fixation by bacteria associated with roots of corn, rice, sugar cane and certain other grasses has been reported[8,9].

Present addresses:
* Department of Agricultural Microbiology, Faculty of Agriculture, University of Ankara, Turkey.
** Laboratory for Nitrogen Fixation Research, Oregon State University, Corvallis, Oregon *97331*.
*** Department of Microbiology, Oregon State University, Corvallis, Oregon *97331*.

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Several investigations have been conducted on the nitrogen fixing capabilities of bacteria in soils from the temperate climates of the world. Anaerobic and facultative anaerobic nitrogen-fixing bacteria were isolated in New Zealand[13], *Enterobacter cloacae* was isolated from the rhizosphere of corn in Oregon[16], and Klebsiella strains from the surfaces of roots and nodules of soybeans, alfalfa and clover were identified[10]. A variety of facultatively anaerobic Enterobacter species have been isolated from rotting wood[1].

In view of the economic importance of wheat throughout the world, this study was carried out for the purpose of isolating and characterizing nitrogen fixing bacteria that possibly may form an effective nitrogen fixing association with wheat.

MATERIALS AND METHODS

Source of isolates

Three areas in the province of Ankara, Turkey were chosen for the field survey. Thirty samples of wheat plants with attached roots were obtained from randomly chosen wheat fields along a 45-kilometer distance on the road from Ankara to Konya. Nine samples were obtained from the University of Ankara, Faculty of Agriculture research field, and six samples from wheat fields along a 50-kilometer distance on the road from Ankara to Haymana. All samples were stored at 4°C in sterile plastic bags.

Wheat roots were washed with sterile tap water, then small pieces of roots 10 to 15 mm long were incubated anaerobically in Hino and Wilson medium. When turbidity in tubes was observed, nutrient agar slants were inoculated from each of the culture tubes. These were forwarded to the Nitrogen Fixation Laboratory, Corvallis, Oregon, where isolates were purified by a plating on Nutrient Agar.

The nitrogen fixing capability and physiological characteristics of the isolates were determined in Hino and Wilson medium modified by the addition of 0.03% yeast extract.

Characterization of the nitrogen fixing isolate

Diagnostic biochemical tests were conducted according to the Manual for Identification of Medical Bacteria[7] and The Manual of Microbiological Methods[6]. Turbidity of cultures were measured with a Bausch and Lomb 340 spectrophotometer at 540 nm. The determination of proteins was carried out by use of the Biuret reaction[11,20]. Bovine serum albumin was used as standard. Numbers of cells were determined from a standard curve relating viable cell counts on nutrient agar plates to optical densities of suspensions at 540 nm.

Acetylene reduction

Ethylene was determined by use of a Hewlett-Packard Model 5830 A gas chromatograph with a flame ionization detector. The chromatograph was equipped with a column (6.4 mm × 1.8 m) of Poropak R and operated at 48°C. The carrier gas was nitrogen used at a flow rate of 35 ml per minute.

Hydrogen production and CO_2 evolution

For the measurement of H_2, a Hewlett-Packard Model 5830 A gas chromatograph with thermal conductivity detector was used. The instrument was fitted with a column (6.4 mm × 1.8 m) of molecular 5A and operated at 120°C. The carrier gas N_2 was used at a flow rate of 35 ml per minute. For the determination of CO_2 samples were assayed by a Carle 1885 gas chromatograph fitted with a thermal conductivity detector and a column (3.2 mm × 45 cm) of Poropak Q at 75°C. The flow rate of the carrier gas helium was 15 ml per minute.

Isolation of deoxyribonucleic acid and determination of the percent guanine plus cytosine DNA base composition

DNA was extracted and purified by modification of the Marmur technique which involves the use of phenol in deproteinization[17]. Percent GC was determined by the thermal denaturation technique[14]. DNA from *E. coli* WP2 was used as the standard.

Growth chamber experiment

An experiment was initiated on January 4, 1980 and continued until February 1, 1980, in a growth chamber with light intensity at 50 cm above the plants of 2200 lux and a day length of 16 hours. The temperature was maintained at 25 ± 1°C. Wheat plants (c.v. Stephens) were grown on agar slants in (200 × 25 mm) test tubes and were supplied with sufficient 1/4 strength nitrogen-free nutrient solution to maintain adequate liquid in the tubes. Seeds were surface disinfected by immersion in 95% alcohol for 30 seconds and then placed in 2% NaOCl for 15 minutes, washed ten times with sterile distilled water (100 ml) and germinated on agar. Seedlings were transferred to test tubes containing nutrient solution in 0.8% agar. At the time of transfer, a group of seedlings was inoculated with a suspension of *Klebsiella oxytoca* ZMK-2, another group with *Azotobacter vinelandii* and a third group with a mixed culture of the two organisms.

Nitrogen content of plants

Total nitrogen in wheat plants was determined by a modified Microkjeldahl technique[21] using NH_4Cl as a standard.

RESULTS

Forty-five bacterial isolates from wheat root were tested for their capacities to reduce acetylene anaerobically in the Hino and Wilson medium. Three of these exhibited appreciable nitrogenase activity. The isolate which showed the highest activity (ZMK-2) was chosen for detailed investigations. For the comparison of some physiological properties of ZMK-2, a series of Klebsiella strains were obtained from a laboratory collection at Oregon State University. These included *Klebsiella pneumoniae* FC 2105, FC 2106, *Klebsiella oxytoca* 2103 and 2104, *Klebsiella oxytoca* V-112 and *Klebsiella* sp. V-233.

Growth and nitrogen fixing capacity

Growth rates, nitrogenase activity (C_2H_2 reduction) and protein contents of isolate ZMK-2 were determined over a five-day culture period (Fig. 1). Maximum rates of ethylene formation occurred during the period of 12 to 24 hours. This corresponds with the period of maximum increase in protein content of cells. Cultures reached the stationary phase of growth after a period of about 40 hours.

Taxonomic properties of the isolate

Microscopic examination showed that the ZMK-2 isolate was a small non-motile rod that was present either as single cells or very short chains. The cells examined at different physiological stages of growth were always gram negative. On the nutrient agar the colonies were smooth, moist, white and easily emulsified in saline. Pigments were not observed on the nutrient agar slant. Utilization of various carbon compounds by isolate ZMK-2 was examined and it was found

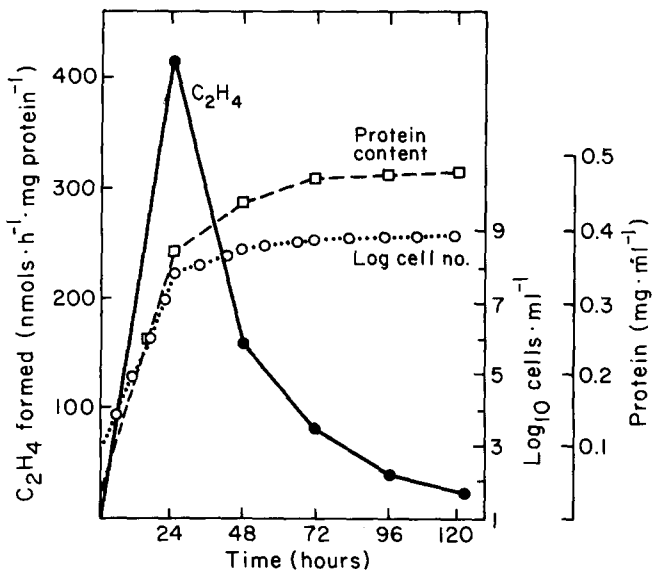

Fig. 1. Protein content, cell number, and C_2H_2 reduction of *Klebsiella oxytoca* during growth of ZMK-2 isolate. Cultures were grown in H-tubes for five days at 30° ± 1 without shaking in yeast extract supplemented Hino and Wilson medium. Each day five samples were removed for growth measurements. To determine C_2H_2 reduction rates, acetylene equal to 10% of the gas volume was injected into H tubes and after five hours of incubation, the ethylene reduction rate was measured and expressed on a cell protein basis. Each point represents the mean of five determinations. Protein content of cells and cell numbers were determined as described in Materials and Methods.

Table 1. Characteristics of isolate ZMK-2

Characteristics	Reaction*
Gram reaction	−
Motility	−
Catalase production	+
Oxidase activity	−
Indol production	+
Methyl-red reaction	+
Voges Proskauer	+
Citrate utilization	+
Urea hydrolysis	+(w)
NO_3^- to NO_2^-	+
Gelatine hydrolysis	+
Starch hydrolysis	−
Pectine hydrolysis	+
TSI agar slant (H_2S production)	+
Arginine dihydrolase	−
Lysine decarboxylase	+
Ornithine decarboxylase	−

* A positive reaction is noted by (+), a negative reaction by (−), and a weak reaction by (+w).

that neither dulcitol nor propionate was used as a carbon source under either aerobic or anaerobic conditions. Some physiological and biochemical characteristics of N_2 fixing ZMK-2 are summarized in Table 1.

H_2 evolution and CO_2 production of isolate ZMK-2 were tested. Increasing production of H_2 and CO_2 were observed under anaerobic conditions during a five-day growth period. In addition, measurements were made of the amount of CO_2 and H_2 evolved from cultures of several strains of Klebsiella provided with either mannitol lactose or dextrose as carbon sources. Most of the strains produced more H_2 than CO_2. *Klebsiella oxytoca* V-112 cultured on a medium containing dextrose, however, produced a greater concentration of CO_2 than H_2 during a 24-hour period (Fig. 2). These results indicate that the relative quantities of CO_2 and H_2 produced by isolates should not be used as a taxonomic characteristic as suggested in Bergey's Manual Determinative Bacteriology[5].

From the deoxyribonucleic acid (DNA) melting curve the GC percentage was found to be 58.3.

Effect of environmental conditions on growth and nitrogenase activity

Acetylene reduction rates by cultures were affected by partial pressures of O_2 in the gas phase. In a series of experiments the maximum rates of ethylene formation were observed at 0.001 atm. O_2. At partial pressures of O_2 of 0.01 and 0.1 atm.

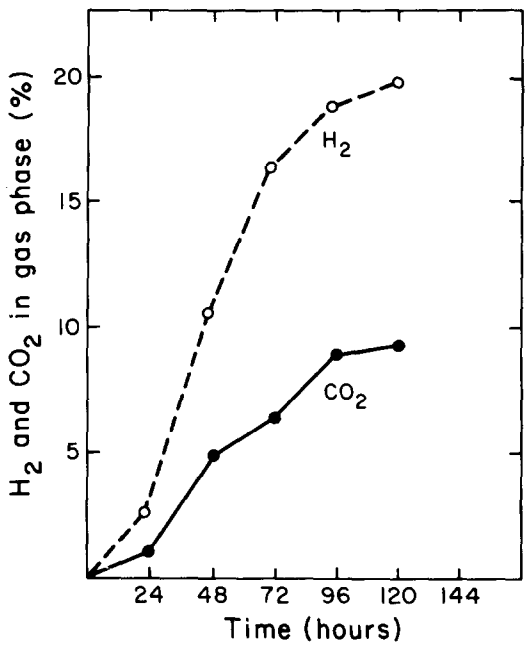

Fig. 2. H_2 and CO_2 production by *Klebsiella oxytoca* ZMK-2 during growth. Cultures were grown for five days on Hino and Wilson's medium under anaerobic conditions at 30°C. Additional experimental conditions are described in the legend of Fig. 1. After growth periods indicated in the figure, samples of gas were removed and assayed for H_2 and CO_2 by procedures described under Materials and Methods.

over the cultures, acetylene reduction was detected but the rate was very low (Fig. 3). Nitrogenase activity also was affected by the partial pressure of H_2 in the gas phase over the cultures (Fig. 4). The maximum rate of ethylene formation was observed in cultures containing 0–2% of H_2 and greater partial pressures of H_2 were increasingly inhibitory. This effect has been observed with many microorganisms[2].

The optimal temperature for growth in nutrient broth was in the range of 30 to 37°C. The highest rates of acetylene reduction were observed at 30°C under anaerobic and detectable acetylene reduction was observed in the range of 10 to 37°C (Fig. 5).

Acidity has been found to be a major factor controlling the abundance of N_2 fixing organisms in the soil. Generally environments that are more acid than pH 6.0 contain none or a very few N_2-fixing microorganisms[2]. In this research the optimum growth and acetylene reduction was observed at pH values ranging from 5.0 to 9.0.

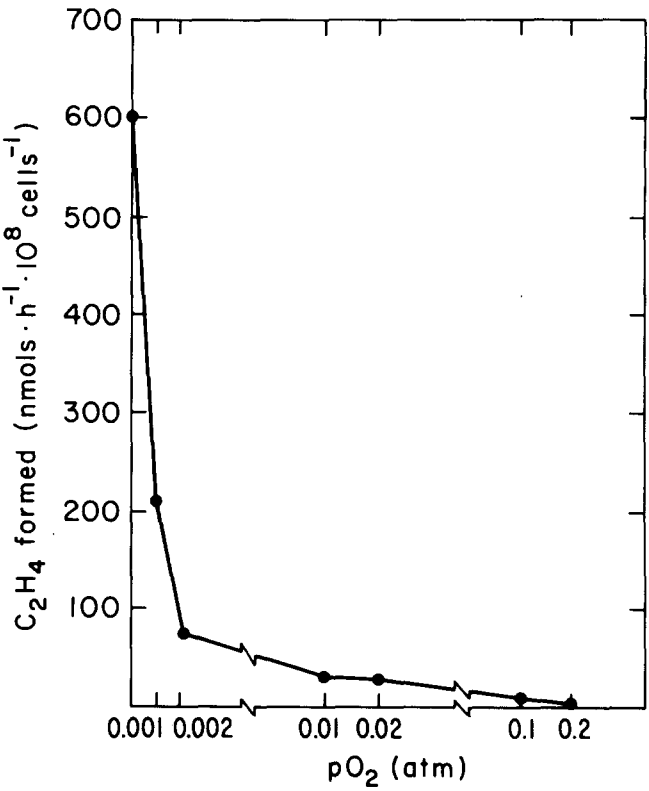

Fig. 3. Effect of partial pressure of O_2 on C_2H_2 reduction by *Klebsiella oxytoca* ZMK-2. Cultures were grown for 24 hours in modified Hino and Wilson's medium at 30°C under anaerobic conditions without shaking. After this the H tubes were then flushed with the N_2 for 20 minutes, a 2-ml sample of each culture was removed and placed in a 25 ml test tube that previously had been filled with a gas mixture containing the indicated amounts of O_2, 10% C_2H_4 and the remainder to 1 atm. with N_2. Four replicate cultures were prepared for each O_2 partial pressure. The tubes were shaken for four hours at 30°C and C_2H_4 formation was measured. Each point on the curve is a mean of determination on four replicate samples.

DISCUSSION

Klebsiella oxytoca has been characterized by indol formation, delayed gelatine liquefaction and a capacity for growth and gas production at low temperatures[19]. In addition to this data, breakdown of pectate and lack of gas production in a medium containing lactose at 44.5°C have been included as characteristics in the taxonomy of this species[18]. No further details on the classification of *Klebsiella oxytoca* is included in Bergey's Manual of Determinative Bacteriology[5]. Generally Klebsiella strains are identified by lack of motility, glucose fermentation with

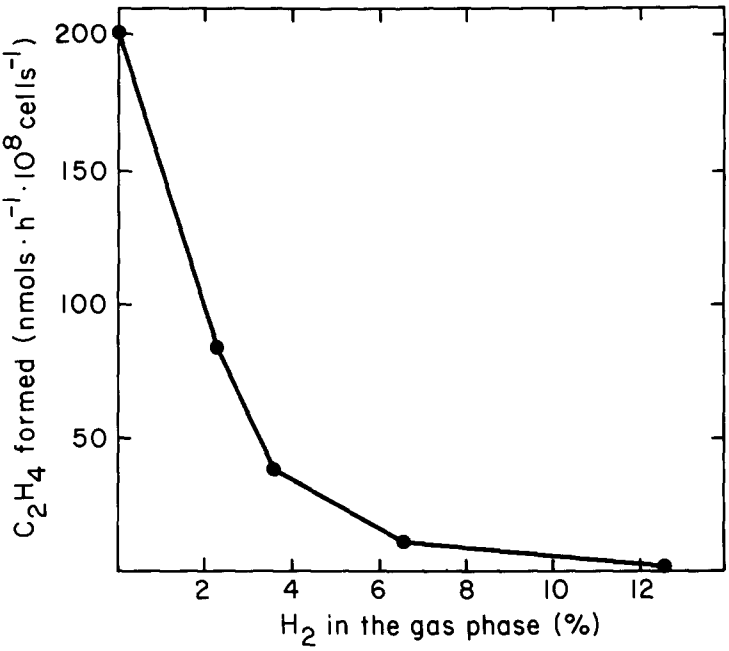

Fig. 4. Effect of the H_2 in the gas phase over the cultures on acetylene reduction by *Klebsiella oxytoca* ZMK-2. Cultures were grown in H tubes at 30 ± 1°C in Hino and Wilson's medium containing 0.05% yeast extract. After 24 hours, H tubes were flushed with nitrogen for 20 minutes and 2 ml samples were removed and placed in 32 ml test tubes that previously were filled with indicated amounts of H_2. Then 0.1 atm. acetylene was added and the tubes were adjusted to 1 atm. with N_2. Three replicate tubes were prepared for each H_2 percentage. The amount of ethylene was measured after three hours incubation at 30°C with shaking on a rotary shaker. Points in the graph are means of three replicate determinations.

production acid and gas (more CO_2 than H_2) and lysine decarboxylase activity.

Based on the 58% G + C DNA base composition and characteristics presented the wheat isolate ZMK-2 appeared to be closely related to *Klebsiella oxytoca.* The production of H_2S on TSI agar is an unusual trail of *Klebsiella* species but was noted before for some wood associated strains[1]. The composition of gas produced by other Klebsiella strains showed more H_2 than CO_2 with one exception which was *Klebsiella oxytoca* V. 112. Acid production has been found to be a factor limiting nitrogenase activity of Klebsiella[24]. For this reason to determine the effect of pH on growth and nitrogenase activity by *Klebsiella oxytoca* ZMK-2 different buffers were used during the experiment. The optimum pH was found to be 6.0.

Another factor limiting nitrogenase activity by *Klebsiella oxytoca* ZMK-2 was H_2 accumulation in the gas phase above culture. This was most evident when the

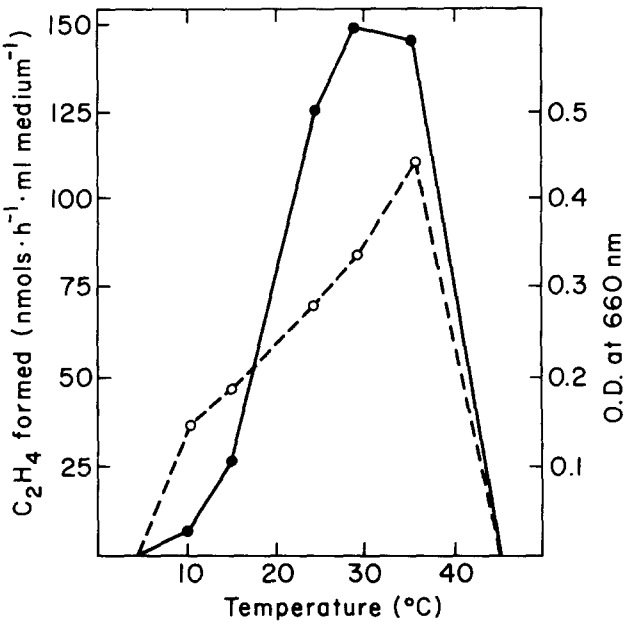

Fig. 5. Effect of temperature on cell mass and nitrogenase activity of *Klebsiella oxytoca* ZMK-2. Optical densities were measured after incubation at temperatures indicated. Acetylene reduction rates were determined on cultures grown in H tubes containing liquid Hino and Wilson's medium supplemented with 0.002% yeast extract. At temperatures of 25, 30 and 37°C cultures were incubated for 24 hours in H tubes and C_2H_2 (10% of gas volume) was added and C_2H_2 produced determined after four hours of incubation. At temperatures of 5, 10, 15 and 45°C cultures were incubated for 48 hours then C_2H_2 was added and C_2H_4 determined as described above. Temperature -- ○ --; C_2H_4 formed— ● —.

H_2 concentration in the gas phase reached more than 2% where nitrogenase activity was reduced more than 70%. Although H_2 is a common end product of anaerobic metabolism little is lost to the atmosphere from flooded fields because it is probably used as a source of energy by the CH_4 producing bacteria. For this reason H_2 accumulation might not be a factor limiting nitrogenase activity under anaerobic conditions in the soil. The effect of O_2 on nitrogenase activity by *Klebsiella oxytoca* ZMK-2 was similar to its effect on the activity in *Klebsiella aerogenes*[12]. Klebsiella that are capable of nitrogen fixation have been isolated from several sources including living wood[1], pulp mill effluents[15], marine environments[23], and surfaces of nodules and roots of legumes[10].

Klebsiella strains under laboratory conditions are known to be capable of nitrogen fixation. We know of no experiment, however, in which the effect of inoculation of plant roots with this organism has been determined. Since we do not know the role of the organism in nature, an experiment was conducted to

study the effect of inoculation of *Klebsiella oxytoca* ZMK-2 on the dry weight and nitrogen content of wheat plants.

Inoculation with live *Klebsiella oxytoca* ZMK-2 and *Azotobacter vinelandii* separately and also as mixed culture of these organisms produced no significant increase in plant growth as measured by plant dry weight. The experiment which was conducted with relatively young wheat plants provided no evidence of an increase in growth due to nitrogen fixation. No growth experiments were conducted in which the environmental conditions were varied in an effort to increase the N_2 fixing capability of *Klebsiella oxytoca* in association with wheat plants. It has been reported that a single bacterial species failed to fix measurable quantities of nitrogen, whereas a combination of species was more effective. Further work on the conditions for optimum N_2 fixation by *Klebsiella oxytoca* obviously needs to be conducted.

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