Isolation of a strain of *Agrobacterium tumefaciens* (*Rhizobium radiobacter*) utilizing methylene urea (ureaformaldehyde) as nitrogen source

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Abstract: Methylene ureas (MU) are slow-release nitrogen fertilizers degraded in soil by microbial enzymatic activity. Improved utilization of MU in agricultural production requires more knowledge about the organisms and enzymes responsible for its degradation. A Gram-negative, MU-degrading organism was isolated from a soil in Sacramento Valley, California. The bacterium was identified as *Agrobacterium tumefaciens* (recently also known as *Rhizobium radiobacter*) using both genotypic and phenotypic characterization. The pathogenic nature of the organism was confirmed by a bioassay on carrot disks. The MU-hydrolyzing enzyme (MUase) was intracellular and was induced by using MU as a sole source of nitrogen. The bacterial growth was optimized in NH_4Cl, urea, or peptone, whereas the production and specific activity of MUase were maximized with either NH_4Cl or urea as a nitrogen source. The result has a practical significance, demonstrating a potential to select for this plant pathogen in soils fertilized with MU.

Key words: methylene urea, ureaformaldehyde, slow-release fertilizer, soil, nitrogen, isolation, *Agrobacterium tumefaciens*, *Rhizobium radiobacter.*

Résumé : Les méthylène-urées (MU) sont des engrais azotés à libération lente qui sont dégradés dans le sol par l'activité enzymatique microbienne. Une meilleure connaissance des organismes et des enzymes responsables de cette dégradation permettrait une utilisation plus efficace du MU en agriculture. Un organisme Gram-négatif dégradant le MU a été isolé d'un sol dans la Vallée de Sacramento en Californie. La bactérie fut identifiée comme étant *Agrobacterium tumefaciens* (aussi connue récemment comme *Rhizobium radiobacter*) à l'aide de caractérisations génotypiques et phénotypiques. La nature pathogène de l'organisme fut confirmée par une bioessai sur des disques de carottes. L'enzyme dégradant le MU (MUase) était intracellulaire, et fut induite par l'utilisation du MU comme seule source d'azote. La croissance bactérienne a été optimisée dans du NH_4Cl, de l'urée ou du peptone, alors que la production et l'activité spécifique de la MUase a atteint un maximum avec soit du NH_4Cl ou de l'urée comme seule source d'azote. Ce résultat a une importance pratique car il souligne le risque de sélectionner en faveur de ce pathogène végétal dans les sols fertilisés avec du MU.

Mots clés : méthylène-urée, urée-formaldéhyde, engrais à libération lente, sol, azote, isolation, *Agrobacterium tumefaciens*, *Rhizobium radiobacter.*

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Introduction

Methylene ureas (MU) are widely used as slow-release fertilizers in professional turf, lawn, plant nurseries, and home gardening applications (Trenkel 1997). They are condensation products of urea and formaldehyde that consist of polymerized chains of various lengths. The degree of polymerization and the combination of different polymer chain lengths in the product determine the slow-release characteristics of different MU. To be available for plant uptake, nitrogen (N) held in the MU polymer has to be hydrolyzed and converted into ammonium ions in the soil. Benefits from using MU as a N fertilizer include more efficient N use by plants, reduced volatilization and leaching losses of N, and prolonged availability of N throughout the growing season (Alexander and Helm 1990). Currently, the estimated use of MU is about 220 000 metric tons per year, mostly in the US, Western Europe, Israel, and Japan (Trenkel 1997), and it is

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projected to increase in the future because of expectations for more efficient fertilizer use in agriculture, horticulture, and forestry.

MU is known to be degraded by microbial activity (Fuller and Clark 1947; Mueller and Heisig 1968). Only recently has more specific information on the organisms capable of hydrolyzing MU been published. Jahns and his co-workers have isolated and identified two MU-degrading Gram-negative organisms in German soil: *Ochrobactrum anthropi* (Jahns et al. 1997) and *Ralstonia paucula* (Jahns and Kaltwasser 2000). From these organisms, they have purified and characterized enzymes (MDUases) that hydrolyze MU to ammonium. However, the detailed mechanism and the intermediates of this hydrolysis still remain unclear as does the role of other organisms and processes responsible for MU degradation in field soils.

The purpose of this study was to isolate and identify soil organisms capable of utilizing MU as a sole N source. One of our main questions was whether organisms isolated by Jahns and his co-workers in Germany (Jahns et al. 1997; Jahns and Kaltwasser 2000) are also responsible for MU degradation in the different soil and climatic conditions prevailing in Sacramento Valley, Calif. This knowledge of the species diversity associated with the degradation of MU is required for understanding and predicting N release from MU in different ecological conditions. In our study, we also optimized growth and MU-hydrolyzing-enzyme production of the isolated organism to produce enough enzyme for future biochemical characterization.

Materials and methods

Preparation of MU

MU (Enduro Long, manufactured by Kemira Agro Oy, Finland) was fractionated according to its water solubility. The method used was based on the Association of Analytical Communities (AOAC) Official Method 945.01 (AOAC 1999). The MU fraction used for enzyme assays contained the cold-water-soluble portion of MU N with no free urea and was prepared by mixing 10 g of MU in 500 mL of H_2O at 25 °C. After 30 min of mixing, the suspension was filtered, and the clear solution containing the cold-water-soluble portion of N (CWSN) was evaporated to dryness. To remove excess free urea in the sample, the powder was dissolved in 50 mL of water at 25 °C, filtered, and the filtrate discarded. The remaining powder (about 0.1 g) was dissolved in 100 mL of H_2O and filtered. The filtrate pH was adjusted to 8.0, and it was incubated with jack bean urease (ICN Biomedicals, Aurora, Ohio) for 24 h. Urease was removed by using a Centriplus 30-kDa microfilter concentrator (Amicon Inc., Beverly, Mass.). The fraction used to isolate MU-degrading organisms was hot-water-insoluble methylene urea nitrogen (HWIN), typically the most resistant to enzymatic hydrolysis. It was prepared by suspending 50 g of commercial MU in 1 L of deionized water and by boiling under stirring for 1 h. The suspension was then filtered, and the MU remaining on the filter (HWIN) was washed twice with 100 °C water and dried at 75 °C.

Isolation of bacteria

A typical field soil in California Sacramento Valley was used for bacterium isolation. The soil is classified as Yolo silt loam (fine-silty, mixed, nonacid, thermic, Typic Xerorthent). A total of 20 subsamples (from 0 to 15 cm depth) were collected in early November from a site that had been fallow for the past 5 years and had no previous history of MU use. Subsamples were mixed together, and the composite soil sample was brought to the laboratory and used immediately for inoculations.

One-gram samples of field soil were used to inoculate six 100-mL batches of a mineral medium (Schlegel et al. 1961) containing 2% *m*/*v* glucose as a carbon (C) source and 0.1% (*m*/*v*) HWIN as a sole N source. Cultures were grown aerobically in an incubator shaker (130 $r \cdot min^{-1}$) at 28 °C for 3 weeks, after which 1.0 mL of each suspension was transferred into 100 mL of fresh medium. At this point, four out of six cultures had no growth, and no growth was observed even after six more weeks of incubation. The two cultures with growth were transferred into fresh media three more times, after which the cells were diluted in 1% (*m*/*v*) NaCl and plated on a same mineral medium with 1.8% (*m*/*v*) agar. Growth was visible after 48 h on the solid medium, and after 72 h, single colonies were isolated, tested for purity on tryptic soy agar, and stored at –80 °C for further experiments.

16S rDNA sequencing and data analysis

Extraction of genomic DNA from MU-degrading bacterial cultures was performed by standard methods (Ausubel et al. 1997). The pellet obtained after cell lysis was suspended in Tris–EDTA buffer, and Microspin columns (Amersham Pharmacia Biotech Inc., Uppsala, Sweden) were used for DNA purification. For PCR amplifications, two universal primers for *Escherichia coli* rDNA 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 907r (5′-CCCGTCAATTCCTTTGAGTTT-3′) were used as sense and antisense primers, respectively. PCR amplifications were carried out in 50-μL reaction volumes with 1 U *Ex Taq*™ polymerase (1 U ≈ 16.67 nkat) (TaKaRa, Fisher Scientific, Pittsburgh, Pa.). Reactions were performed for 1 min at 94 °C, then cycled 30 times (1.5 min at 94 °C, 1.5 min at 57 °C, 2 min at 72 °C), and then the extension reactions were carried out for 10 min at 72 °C. Amplification products were cloned into *E. coli* competent cells by using a TOPO TA Cloning kit (Invitrogen, Carlsbad, Calif.). Transformant plasmids were extracted and purified by using the Qiagen plasmid minipurification protocol (Qiagen Inc., Valencia, Calif.). The double-stranded DNA was used as template for sequencing, using both the 27f and 907r primers. Nucleotide sequencing was carried out by Davis Sequencing, LLC (Davis, Calif.) by using an ABI PRISM 3100 DNA sequencer. BLAST 2.2.1 algorithm (Altschul et al. 1990) at the National Center for Biotechnology Information (NCBI) was used for alignment of nucleotide sequences with information from GenBank, EMBL, DDBJ, and PDB databases.

Phenotypic characterization

Phenotypic characterization of a pure bacterial culture was performed according to *Bergey's Manual of Determinative Bacteriology* (Holt et al. 1994). Tests suggested by Moore et al. (1988), Sawada and Ieki (1992), and Young et al. (2001)

for differentiation among different *Rhizobium* species were used for characterization. Methods were adopted from Keane (1970), Holmes et al. (1975), and Moore et al. (1988) unless otherwise indicated. Antibiotic resistance tests were performed according to Bizet and Bizet (1995) and Higgins et al. (2001) by using antibiotic disks (Becton Dickinson Microbiology Systems, Cockeysville, Md.) on a Mueller–Hinton medium. The following phenotypical features were analyzed: (*i*) Gram stain and motility at room temperature; (*ii*) catalase and oxidase production; (*iii*) utilization of lactose, mannitol, and soluble starch as sole C sources; (*iv*) growth on MacConkey and Luria–Bertani agar; (*v*) growth and pigmentation in ferric ammonium citrate; (*vi*) growth at 28 and 37 °C; (*vii*) fluorescence on Kings B agar; (*viii*) tolerance to Kanamycin (30 $\mu g \cdot mL^{-1}$), Cefuroxime (30 $\mu g \cdot mL^{-1}$), Penicillin G (10 IU), and Tetracycline (30 $\mu g \cdot mL^{-1}$); (*ix*) production of 3-ketolactose (Bernaerts and De Ley 1963), and (*x*) production of β-galactosidase (ONPG: *o*-nitrophenyl-β-D-galactopyranoside). Carbohydrate utilization pattern for the isolated organism was also tested by using the BIOLOG420 program for Gram-negative, non-enteric bacteria (BIOLOG, Hayward, Calif.).

Bioassay for testing phytopathogenicity

The ability of MU-degrading bacterium to infect plants and produce galls was tested in a carrot disk bioassay (Ark and Schroth 1958; Lippincott and Lippincott 1969) by using a protocol of Moore et al. (1988). In this test, bacterial suspension was added to the surface of freshly cut carrot disks. Disks were kept moist in a sterile Petri dish and incubated at room temperature. After 3 weeks, the disks were checked for the formation of young galls (tumors) developing from the meristematic tissue around the central vascular system. *Agrobacterium tumefaciens* cultures obtained from Dr. L. Epstein at the University of California, Davis, Department of Plant Pathology, were used as positive (*A. tumefaciens* (Smith and Townsend 1907) Conn 1942 strain 20W16C, bv. 1) and negative (*A. tumefaciens* (Smith and Townsend 1907) Conn 1942 strain 20W16B, bv. 2) controls.

Preparation of crude extracts

For the preparation of crude extracts, cells were grown in the mineral medium to an early stationary phase and harvested by centrifugation (3500*g* for 15 min at 4 °C). The pellet was washed twice in 50 $mmol \cdot L^{-1}$ Na_2HPO_4 (pH 7.5) containing 3 $mmol \cdot L^{-1}$ mercaptoethanol. Cells (0.2 g wet weight·(mL of buffer)$^{-1}$) were disintegrated by an ultrasonic treatment (Sonic 60 dismembrator, Fisher Scientific, Inc., St. Louis, Mo.) at 30 W for 30 $s \cdot mL^{-1}$. Sonication was performed in 10-s pulses with cooling in an ice bath. Broken cell suspensions were centrifuged (8500*g* at 15 °C for 20 min), and the supernatants were used for enzyme assays and protein concentration determinations.

Enzyme and protein assays

Activity assays for the MU-degrading enzyme (MUase) were performed in 25 $mmol \cdot L^{-1}$ Na_2HPO_4 (pH 8.0), 1.5 $mmol \cdot L^{-1}$ mercaptoethanol, and 0.5 $mol \cdot L^{-1}$ EDTA. After 1 h of incubating the enzyme preparation with 1 $mmol \cdot L^{-1}$ MU (CWSN) at 30 °C, the amount of ammonium produced was determined by using a colorimetric reaction based on the Berthelot reaction (Forster 1995). In this method, 5–40 μL of assay solution was mixed with 0.5 mL of salicylate-nitroprusside color reagent and 0.5 mL of alkaline NaOH-sodium hypochlorite oxidizer. After incubation at 30 °C for 15 min, absorbance was measured with a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corp.) at 660 nm. A treatment without enzyme was used in all assays as a control for substrate ammonium content. Protein content in the crude extract was measured by the Bradford method (Bradford 1976, Bio-Rad microassay, Bio-Rad, Hercules, Calif.) by using bovine serum albumin as a standard. Enzyme activities are expressed as units (U) with 1 U corresponding to 1 μmol NH_4 produced per minute.

Optimization of bacterial growth and enzyme production

Since bacterial growth in a liquid medium with only MU as a N source was extremely slow, other types of N sources were tested to promote faster growth and enzyme production. Besides MU, ammonium chloride and peptone (Kings B medium) were used as N sources. For growth optimization, each culture (100 mL) was inoculated with 1.0 mL of bacteria grown in Kings B medium. Cultures were grown at 28 °C for times specified in each treatment, after which the cells were harvested and made into crude extracts. Optimization for enzyme production was performed in liquid mineral medium containing MU (HWIN), NH_4Cl, urea, KNO_3, or peptone as a sole source of N. The concentration of N in all other media except the peptone medium was 18 $mmol \cdot L^{-1}$. Kings B medium was used as peptone source, and its peptone content was 20 $g \cdot L^{-1}$. Media (100 mL) were inoculated with 1.0 mL of bacterial culture in MU and grown in an incubator shaker at 28 °C for 7 days. During the growth period, the growth curve of each culture was determined by measuring the optical density (OD) with a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corp.) at 600 nm. Cells in each culture were harvested, and crude extracts were prepared for use in enzyme and protein assays. Each treatment was carried out in three replicates, and the numbers presented here are averages of those replicates.

Results

Isolation of bacterium

During bacterial isolation from a typical Sacramento Valley field soil, both cultures yielded identical isolates, and only one type of colony was detected in plates containing MU as a N source. The colonies of this MU-degrading bacterium were white, circular, convex, and appeared after 48 h of incubation at 28 °C. Interestingly, when growing in the liquid medium, the bacterium produced large amounts slime, making the culture very viscous.

Identification of bacterium

The 16SrDNA nucleotide sequence from the MU-degrading bacterium isolated in this study is posted in the international database with GenBank acc. No. AF498042. The BLASTN search indicated that the organism belongs to *Proteobacteria*, α subdivision, *Rhizobiaceae* group. According to the BLASTN analysis, the 16S rDNA nucleotide sequence from the MU-degrading bacterium was 99% identical to corresponding sequences from *Rhizobium* sp. NM349 (AF345545; Gao et al.

Table 1. Phenotypic characteristics of the isolated bacterium.

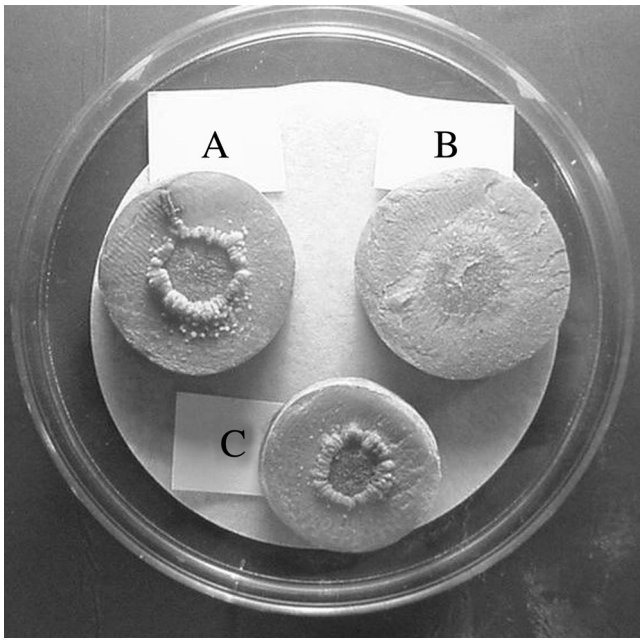
Characteristic	Response
Gram stain	–
Motility at room temperature	+
Production of:	
Catalase	+
Oxidase	+
β-Ketolactose	+
β-Galactosidase	+
Utilization of:	
N_2 as a nitrogen source	–
Methylene urea as a carbon source	–
Lactose	+
Mannitol	+
Starch	–
Growth on:	
MacConkey agar	+
Luria–Bertani agar	+
Ferric ammonium citrate	+
Growth at:	
28 °C	+
35 °C	+
Fluorescence on Kings B agar	–
Resistance to:	
Kanamycin (30 $\mu g \cdot mL^{-1}$)	–
Cefuroxime (30 $\mu g \cdot mL^{-1}$)	–
Penicillin G (10 IU)	+
Tetracycline (30 $\mu g \cdot mL^{-1}$)	+

2001) and several strains of *A. tumefaciens* (ATU38899–ATU389909; Mougel et al. 1999).

Results from the phenotypic tests are presented in Table 1. Based on the phenotypic tests, the bacterium was classified as *A. tumefaciens* (also known as *Rhizobium radiobacter*). Growth without growth factors (biotin, pantothenate, thiamin) as well as growth on MacConkey agar, growth and pigmentation on ferric ammonium citrate, and production of 3-ketolactose differentiated it from other species of Rhizobia, especially from its closest relative, *Rhizobium rhizogenes* (Holmes and Roberts 1981; Moore et al. 1988; Young et al. 2001). Production of β-galactosidase and susceptibility to Kanamycin and Cefuroxime proved that the organism was not *Ochrobactrum anthropi* (Bizet and Bizet 1995; Higgins et al. 2001). According to BIOLOG, the test organism was identified as *Rhizobium radiobacter* with a probability of 95%.

Bioassay with carrot disks showed phytopathogenicity after 3 weeks of incubation (Fig. 1). Both *A. tumefaciens* 20W16C, bv. 1 (positive control) and the MU-degrading test bacterium induced tumor-like callus formation on carrot disks. No gall was detected in disks inoculated with the negative control (*A. tumefaciens* 20W16B, bv. 2). However, the ability to degrade MU does not seem to be ubiquitous in the genus *Agrobacterium*. In a follow-up study, 16 strains of Agrobacteria (biovar 1 and biovar 2) were tested for growth on MU N, and only one (53-1C, biovar 1) was able to grow on MU (data not shown).

Fig. 1. Phytopathogenicity test on carrot disks. Growth of gall (tumor) around the central vascular system indicates phytopathogenicity. (A) Positive control, *Agrobacterium tumefaciens* bv. 1. (B) Negative control, *Agrobacterium tumefaciens* bv. 2. (C) Methylene-urea-degrading bacterium from field soil in Sacramento Valley, Calif.



Optimization of growth and enzyme production

Growth of the isolated bacteria was slow in MU. The growth curve presented in Fig. 2 shows slow linear growth during the first 150 h. However, the growth rate was increased when MU was substituted by other N sources (Fig. 3). Peptone and NH_4Cl as N sources resulted in fast growth and increased production of cell biomass, but without MU the activity of the MUase in the crude extracts was extremely low (Table 2). When cells were first grown in a soluble N source for 4 days and then washed and transferred into a MU medium, they showed a rapid increase in MUase activity. These results indicate that in the test organism, MUase is induced by MU. Jahns et al. (1997) found that production of a MU-hydrolyzing enzyme (MDUase) in *Ochrobactrum anthropi* was partially depressed by ammonium in the growth medium. However, high ammonium concentration did not seem to depress MU-hydrolyzing activity in the MU-degrading bacteria isolated in this study. Cells grown in MU showed a slow increase in enzyme activity until they reached the stationary growth phase. Interestingly, activity never reached the amount produced by cells that were transferred to MU from NH_4Cl or peptone. This observation suggests that either production or inactivation of MUase in bacterial cells is dependent on their nutritional status.

The formation of MUase in MU-induced cells depended strongly on the growth conditions (Table 3). Total activity in cells harvested after 7 days was highest in the NH_4Cl medium, followed by urea and peptone. Specific activity was highest in cells grown with NH_4Cl as a sole source of N. Growth of the isolated bacteria was not optimal with KNO_3.

Fig. 2. Growth of *Agrobacterium tumefaciens* (*Rhizobium radiobacter*) ME1 on methylene urea. Coefficient of variation % of three replicate measurements was less than 12%.

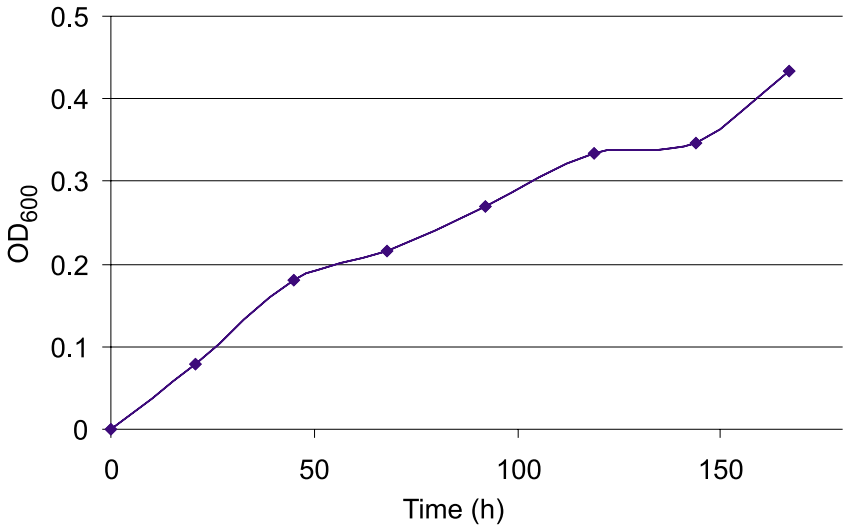
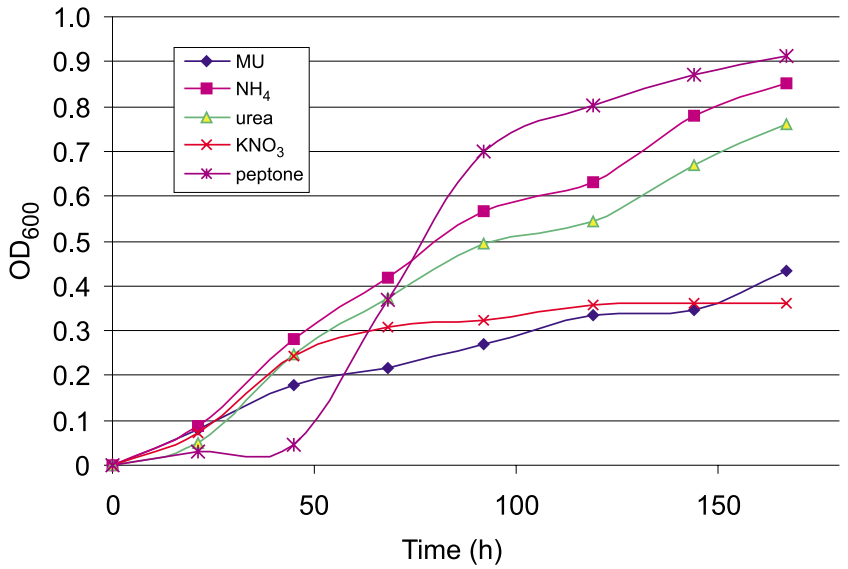


Fig. 3. Growth of *Agrobacterium tumefaciens* (*Rhizobium radiobacter*) ME1 on different nitrogen sources. Coefficient of variation % of three replicate measurements was less than 14%.



The cells reached a stationary growth phase only after 68 h of incubation (Fig. 3), and hence, the protein content in these cells was about 50% lower than in cells grown with other forms of soluble N. In all growth conditions, less than 5% of the total MUase activity was found in the growth medium, which indicated that the enzyme was intracellular.

Discussion

In this study, we were able to isolate a MU-degrading bacteria from a typical field soil in Sacramento Valley, Calif. The MU-degrading strain (ME1) was capable of producing an enzyme (MUase) needed for degradation of MU and, hence, had the ability to use MU as a sole N source for growth. Bacterial identification was carried out by using both genotypic and phenotypic characterization and was supported by a bioassay for phytopathogenicity. The two cultures tested yielded identical isolates. The results strongly suggested that the isolated bacterium is a strain of *A. tumefaciens*. A change in nomenclature and taxonomic classification of *A. tumefaciens* was recently proposed by Young et al. (2001) but the new name, *Rhizobium radiobacter*, has not been decisively accepted (Farrand et al. 2003; Young et al. 2003).

Agrobacterium tumefaciens (*Rhizobium radiobacter*) is an indigenous soil bacterium known for its phytopathogenic effects. It causes crown gall disease in a wide range of dicotyledonous plants, especially members in the *Rosaceae* family, such as apple, pear, peach, cherry, almond, raspberry, and roses (Sigee 1993). The disease is characterized by tumor-like swellings (galls) that typically occur at the crown of the plant, just above the soil level. Upon infection, the bacterium transfers part of its plasmid DNA to the plant. This DNA integrates into the plant's genome, causing the production of

Table 2. Effect of nitrogen source and incubation time on the growth and methylene urea (MU) hydrolyzing enzyme activity of *Agrobacterium tumefaciens* (*Rhizobium radiobacter*) ME1.

Nitrogen source	Time[a] (h)	OD_{600}	Activity (U[b])	Protein[c] (mg)	Specific activity (U·(mg protein)$^{-1}$)
MU	120	0.7±0.1	35±1.7	1.4±0.1	25.0±3.2
	240	0.9±0.1	92±6.2	4.1±0.7	22.4±4.6
	500	1.7±0.2	78±3.6	5.1±0.3	15.3±1.6
NH_4Cl	120	1.1±0.1	2.8±0.2	5.0±0.3	0.6±0.1
Peptone	120	1.3±0.1	1.7±0.1	5.4±0.4	0.3±0.1
NH_4Cl/MU	96/72	na	126±2.6	5.1±0.4	24.7±2.6
Peptone/MU	96/72	na	120±5.5	5.6±0.4	21.4±2.7

Note: In treatments NH_4Cl/MU and peptone/MU, cells were first grown to early stationary phase in either NH_4Cl or peptone, then washed twice with mineral medium without nitrogen, and transferred into medium with MU. na, not analyzed.

[a]Cells were grown at 28 °C for times indicated in the table.

[b]One unit (U) of activity corresponds to 1 µmol NH_4 produced per minute.

[c]Protein was measured in the crude extract after cell lysis.

Table 3. Effect of nitrogen source on methylene urea (MU) hydrolyzing enzyme activity in MU-induced cells of *Agrobacterium tumefaciens* (*Rhizobium radiobacter*) ME1.

Nitrogen source	Total activity (U)	Total protein (mg)	Specific activity (U·(mg protein)$^{-1}$)
MU	80±2.6	3.0±0.2	26.7±2.8
NH_4Cl	199±5.4	5.2±0.6	38.3±4.9
Urea	158±4.9	4.9±0.5	32.3±4.0
KNO_3	59±1.3	2.8±0.1	21.0±2.1
Peptone	153±4.3	5.6±0.4	27.4±2.8

Note: Concentration of nitrogen in each medium was 18 mmol·L^{-1}, except for peptone, which was used as 20 g·L^{-1} of Kings B medium. Cells were harvested after 7 days of incubation at 28 °C.

tumors and associated changes in the plant metabolism. One of these changes is the production of novel plant metabolites, opines, by the infected plant. Opines are unique amino acid derivatives that only the tumor-inducing bacteria can utilize as C and N sources (Dessaux et al. 1992). Agrobacteria are known to be able to utilize a wide variety of recalcitrant substrates, which in some cases first involves transporting the complex molecules into the cytoplasm. One example of the unique catabolic activity of *A. tumefaciens* is its capability to hydrolyze D,L-substituted hydantoins into optically pure D-amino acids, which can be utilized for side chains in semisynthetic antibiotics (Syldatk et al. 1990).

The enzyme MUase was specifically induced by MU, after which its production was not repressed by moderate concentrations of ammonium, urea, or peptone in the growth medium. This is different from the regulation of MU-hydrolyzing enzyme of *Ralstonia paucula* reported by Jahns and Kaltwasser (2000), in which the enzyme was repressed by the presence of ammonium and derepressed in its absence. Tolerance for high soluble N concentrations has a practical aspect in cases where MU is used in combination with other N fertilizers under field conditions. In fact, a readily available N source might even be beneficial to the MUase activity, which according to our study is low in a N-limited bacterial population. However, the results indicate that the test organism does not grow well with nitrate. Poor utilization of nitrate as a sole source of N can be explained by the lack of nitrate or nitrite reductase in this particular strain of *A. tumefaciens*. According to Holmes and Roberts (1981), strains of *A. tumefaciens* vary in their ability to reduce nitrate and (or) nitrite to ammonium.

Growing MUase-induced cells at 28 °C in a mineral medium containing ammonium, urea, or peptone as a N source maximized the production of MUase enzyme by the isolated bacteria. The highest specific activity of MUase was obtained in cultures grown with NH_4Cl as a source of N. For maximum enzyme activity, cells should be harvested at the end of exponential growth phase or at the early stationary growth phase because later on there is a reduction in specific activity. The effect of nutritional status on production and inactivation of specific enzymes by bacteria has been shown earlier by Janssen et al. (1982). In their study, allantoinase inactivation in *Pseudomonas aeruginosa* occurred in stationary growth phase under C or N starvation. Allantoinase catalyses the hydrolysis of allantoin, a purine catabolism intermediate having structural analogies with MU.

Accelerated degradation of MU in soils that have been exposed to MU in the past has been proposed by Hadas et al. (1975) and Waddington et al. (1976). They were able to show in field experiments that continuous use of MU resulted in faster mineralization of MU N over time. They suggested that this was an indication of an increase in the MU-degrading microbial population in the soil. According to our study, MUase in *A. tumefaciens* ME1 is intracellular and is pro-

duced in optimal nutritional conditions. If the presence of actively growing bacteria were a requirement for high MUase activity, then the accelerated degradation of MU in soils with previous MU use would, indeed, be a result of a larger population of MU-degrading organisms. On the other hand, a constant supply of MU is not required for high activity because after induction the enzyme is expressed even in the absence of MU. Hence, the capability of a microbial population to degrade MU may persist in soil without continuous supply of the substrate.

This is the first time *Agrobacterium* sp. has been shown to degrade MU in soil. So far, Jahns and his co-workers have reported that two other Gram-negative bacteria, *Ochrobactrum anthropi* (Jahns et al. 1997) and *Ralstonia paucula* (Jahns and Kaltwasser 2000) isolated in Germany, produce enzymes needed for MU degradation. The finding that a plant pathogen, *A. tumefaciens* (*R. radiobacter*), is responsible for MU breakdown has a practical significance. As the use of MU on container nursery stock and agricultural crops increases (Trenkel 1997), it is important to know its long-term effects on the soil microbial population. It can be very crucial if MU used as a fertilizer changes the microbial diversity in the soil towards bacteria, which are known to be harmful to plants.

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