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Metabolism of Melamine by *Klebsiella terragena*

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Experiments were conducted to determine the pathway of melamine metabolism by *Klebsiella terragena* (strain DRS-1) and the effect of added NH_4^+ on the rates and extent of melamine metabolism. In the absence of added NH_4^+, 1 mM melamine was metabolized concomitantly with growth. Ammeline, ammelide, cyanuric acid, and NH_4^+ accumulated transiently in the culture medium to maximal concentrations of 0.012 mM, 0.39 mM, trace levels, and 0.61 mM, respectively. In separate incubations, in which cells were grown on either ammeline or ammelide (in the absence of NH_4^+), ammeline was metabolized without a lag while ammelide metabolism was observed only after 3 h. In the presence of 6 mM added NH_4^+ (enriched with 5% ^{15}N), ammeline, ammelide, and cyanuric acid accumulated transiently to maximal concentrations of 0.002 mM, 0.47 mM, and trace levels, respectively, indicating that the added NH_4^+ had little effect on the relative rates of triazine metabolism. These data suggest that the primary mode of melamine metabolism by *K. terragena* is hydrolytic, resulting in successive deaminations of the triazine ring. Use of ^{15}N-enriched NH_4^+ allowed estimates of rates of triazine-N mineralization and assimilation of NH_4^+-N versus triazine-N into biomass. A decrease in the percent ^{15}N in the external NH_4^+ pool, in conjunction with the accumulation of ammelide and/or triazine-derived NH_4^+ in the culture medium, suggests that the initial reactions in the melamine metabolic pathway may occur outside the cytoplasmic membrane.

Melamine (2,4,6-amino-*s*-triazine) and related triazines have been proposed as alternative forms of fertilizer-N for plant growth, due to their high N content, low rates of solubilization, and low leaching potential in soil (1, 6, 7, 13). The effectiveness of triazines as fertilizers, however, is dependent upon the presence of microorganisms in the soil that are capable of metabolizing triazines to readily available nitrogen compounds (i.e., urea and/or NH_3). Previous studies suggest that rates of melamine metabolism in soil, even when inoculated with melamine-degrading strains, may be insufficient to support plant growth (7, 13). Consequently, the successful use of triazine fertilizers will require the identification of inoculant strains that degrade melamine in soils at rates sufficient to ensure adequate plant growth.

Relatively little information regarding the microbial metabolism of melamine is available. Cook and Hutter (4, 5) described six strains of bacteria (including three *Pseudomonas* isolates, two *Klebsiella* isolates, and a *Rhodococcus* isolate) that mineralized various *s*-triazine compounds, utilizing them as a source of N for growth. Most strains metabolized various mono-alkylated-, hydroxy-, and/or amino-triazines; however, only *Pseudomonas* strain A and *Rhodococcus corallinus* were capable of growth with melamine. Studies by Jutzi et al. (8) with *Pseudomonas* strain A indicated that melamine was metabolized by successive deamination of the triazine ring, followed by hydrolysis (Fig. 1). Recently, several investigators have reported the isolation and characterization of bacteria capable of the dealkylation of atrazine and subsequent mineralization of the *s*-triazine ring (12, 14, 16). Only one strain was tested for melamine mineralization (14), albeit unsuccessfully. Leeson et al. (11) have described a *Klebsiella terragena* strain (DRS-1) which utilized the triazine 2-chloro-4,6-diamino-*s*-triazine (CAAT; the primary ozonation product of atrazine) as an N source for growth. This strain was observed to metabolize CAAT even in the presence of high concentrations of exogenous NH_4^+; by comparison, CAAT metabolism by *Pseudomonas* strain A was inhibited by exogenous NH_4^+. Strain DRS-1 has also been shown to metabolize melamine (5a) and to stimulate plant growth when used as a component of an inoculant mixture in the presence of melamine (8a).

The purpose of this study was to elucidate the pathway of melamine metabolism by *K. terragena* DRS-1 and to assess the effect(s) of exogenous NH_4^+ on relative rates and extent of melamine-triazine metabolism.

MATERIALS AND METHODS

Culture and media. *K. terragena* DRS-1 has been previously described (11). Cultures were grown at 30°C on NFB medium (15) containing 40 mM glycerol and 1 mM melamine. Melamine was added to the medium after autoclaving from a filter-sterilized 10 mM stock made in water and adjusted to a pH of 7 with HCl.

Incubations. One liter of medium, with or without 6 mM NH_4^+ [with 5% ^{15}N label as $(NH_4)_2SO_4$], was inoculated with 50 ml of a starter culture grown to late logarithmic-early stationary phase. The culture was incubated at 30°C on a rotary shaker (150 rpm). Every half hour, 6-ml samples of culture were centrifuged at high speed in a microcentrifuge (Microfuge 11; Beckman Instruments Inc., Fullerton, Calif.) and the supernatant fraction was decanted into Eppendorf tubes and placed in a heating block (120°C) for 4 min and then frozen until analysis for triazines and NH_4^+. On the hour, cell pellets were washed twice in 20 mM phosphate buffer, resuspended in 0.5 to 1.5 ml of 0.5 N NaOH, and frozen until protein analysis. On the half hour, turbidity was measured with a Klett-Sommerson turbidometer (Klett Manufacturing Co., New York, N.Y.). For the culture grown in the presence of 6 mM added NH_4^+, on the half hour, cell pellets were washed twice and resuspended in 0.5 to 1.5 ml of distilled and deionized H_2O for $^{15}N/^{14}N$ analysis.

In separate incubations, media containing 40 mM glycerol and either 1.5 mM ammeline or 1.5 mM ammelide were inoculated with melamine-grown cells. Every 3 h, 1-ml samples of culture were centrifuged and the supernatant fraction was analyzed for ammeline or ammelide.

Triazine analysis. Melamine, ammeline, and ammelide were analyzed by high-pressure liquid chromatography (HPLC) using a Resolve C_{18} radially compressed column (0.8 by 10 cm; Waters, Milford, Mass.). The solvent system was composed of 13.3 mM heptane sulfonic acid (sodium salt) and 132.4 mM diethylamine in 0.8 N H_3PO_4 (final pH of 2.0); separation was accomplished at a flow rate of 2 ml min^{-1}. Triazines were detected with a Waters 996 photodiode array detector set at 210 nm and quantified based on standard curves. Cyanuric acid

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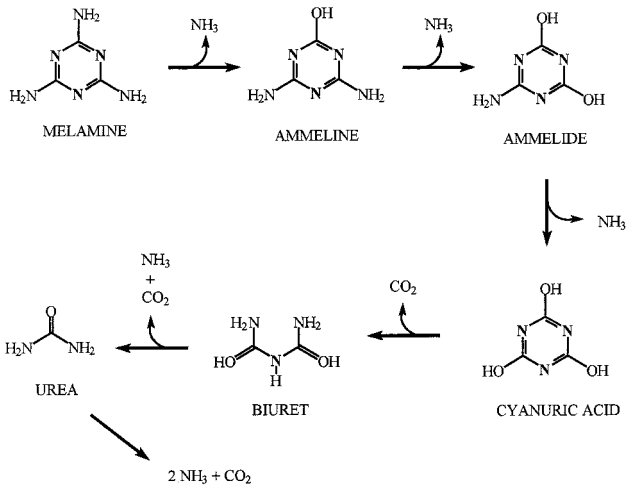

FIG. 1. Pathway of melamine metabolism by *Pseudomonas* strain A and presumptive pathway of melamine metabolism by *K. terragena.*

was analyzed with the same system and under the same conditions, except that the solvent consisted of 5 mM octyltriethylammonium phosphate in 5 mM potassium phosphate (final pH of 6.8) and the detector was set at 214 nm.

N analysis. Ammonium was measured colorometrically by flow injection analysis (Lachat Instruments, Milwaukee, Wis.). The ^{15}N contents of the NH_4^+ and biomass pools were measured by mass spectrometry (MS) using an automatic N-C analyzer interfaced with an isotopic ratio mass spectrometer (Europa Scientific Ltd., Crewe, United Kingdom). These analyses involved Dumus combustion of samples encapsulated in tin (Sn) foil, with isotopic measurement of the N_2 produced by MS. Samples of NH_4^+ from the culture supernatant were prepared by the diffusion techniques of Brooks et al. (3). Samples of biomass were prepared by pipetting an aliquot of suspended biomass into an Sn foil capsule and drying overnight at 60°C. The MS was standardized by using $(NH_4)_2SO_4$ with different certified contents of ^{15}N. Measurements of the size and isotopic composition of the NH_4^+ pool were used to calculate rates for mineralization of triazine-N and biomass assimilation of N from the labeled NH_4^+ and triazine-derived pools of N. These calculations were based on a variation of the equations first derived by Kirkham and Bartholomew (9) for measurement of the gross rates of N transformation in soil. Such calculations provide estimates for transformation rates of a pool of mineralized N that has equilibrated with the ^{15}N-labeled NH_4^+ pool. Measurements of the ^{15}N content of the biomass pool permitted calculations of the contribution of the triazine-derived N pool at natural abundance (0.366%) and of the ^{15}N-labeled NH_4^+ pool into biomass N. These calculations were based on the use of isotope dilution equations and the principle of conservation for the ^{15}N in the system.

Protein analysis. Protein concentrations in the dissolved cell pellets were determined by the method of Bradford (2) using the Bio-Rad (Richmond, Calif.) protein assay, with immunoglobulin G as the standard.

RESULTS

In the absence of added NH_4^+, 1 mM melamine was metabolized to a level below the detection limit (ca. 1 μM) within 6.5 h (Fig. 2). Ammeline accumulated transiently to a maximum of 12 μM (1.5 to 2.5 h) but was metabolized to a level below the detection limit (ca. 1 μM) within 5.5 h (data not shown). Ammelide accumulated transiently to a maximum of 0.39 mM (6 to 6.5 h) followed by metabolism to <1 μM by 9.5 h (Fig. 2). Cyanuric acid was detected at trace levels between 8 and 10 h. Ammonium accumulated in the culture medium to 0.61 mM (4.5 h), followed by rapid dissipation to <0.5 mM, and was below detectable levels (ca. 0.01 mM) by 9 h (Fig. 1). Biomass and protein increased exponentially for 9 h, after which the protein level was constant; the final protein concentration was 1.13 mg ml^{-1} (Fig. 2). Turbidity continued to increase at an approximately linear rate until the end of the incubation (13 h).

In separate incubations, melamine-grown cells inoculated into media containing ammeline as the sole N source metabolized ammeline without a lag (data not shown). However, cells inoculated into media containing ammelide as the sole N source exhibited a 3-h lag before appreciable ammelide metabolism was observed (data not shown).

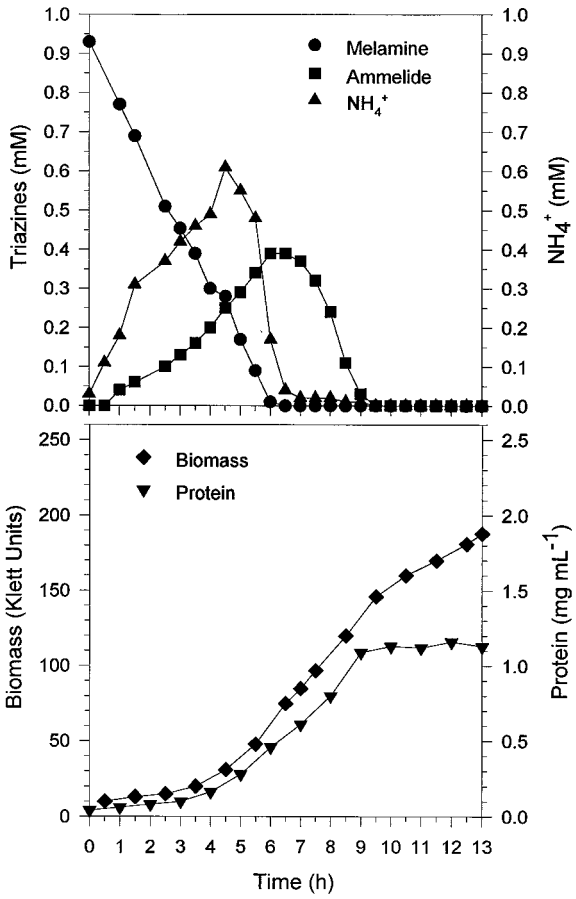

FIG. 2. Growth of *K. terragena* with melamine as the sole N source. The upper panel shows the concentrations of melamine, ammelide, and NH_4^+; the lower panel shows turbidities and protein concentrations.

In the presence of 6 mM added NH_4^+ (enriched with 5% ^{15}N), melamine was metabolized to a level below the detection limit within 6.5 h (Fig. 3). Ammeline accumulated transiently to a maximum of 2 μM but was metabolized to a level below the detection limit within 5.5 h (data not shown). Ammelide again accumulated transiently to a maximum of 0.47 mM (6 h) followed by metabolism to <1 μM by 10 h (Fig. 3). Cyanuric acid was detected at trace levels at 10 h. Ammonium concentrations fluctuated between 6 and 6.14 mM for the first 4 h, followed by rapid dissipation to <0.01 mM by 11.5 h (Fig. 3). Biomass and protein increased exponentially for 11 h, after which protein was constant while biomass increased slightly; final protein concentration was 2.40 mg ml^{-1} (Fig. 3).

The atoms percent ^{15}N of both NH_4^+ and biomass decreased throughout the incubation; the final biomass percent ^{15}N was 2.66 (Fig. 4). Biomass levels were too low prior to 4.5 h, while NH_4^+ concentrations were too low after 9.5 h, to obtain accurate ^{15}N determinations. Rates of NH_4^+ mineralization from triazines increased to a steady state of ca. 0.5 mM h^{-1} for the first 6 h and then decreased concomitantly with ammelide accumulation (Fig. 4). Rates of NH_4^+ assimilation (including both added NH_4^+ and triazine-derived NH_4^+) increased initially concomitantly with growth but subsequently

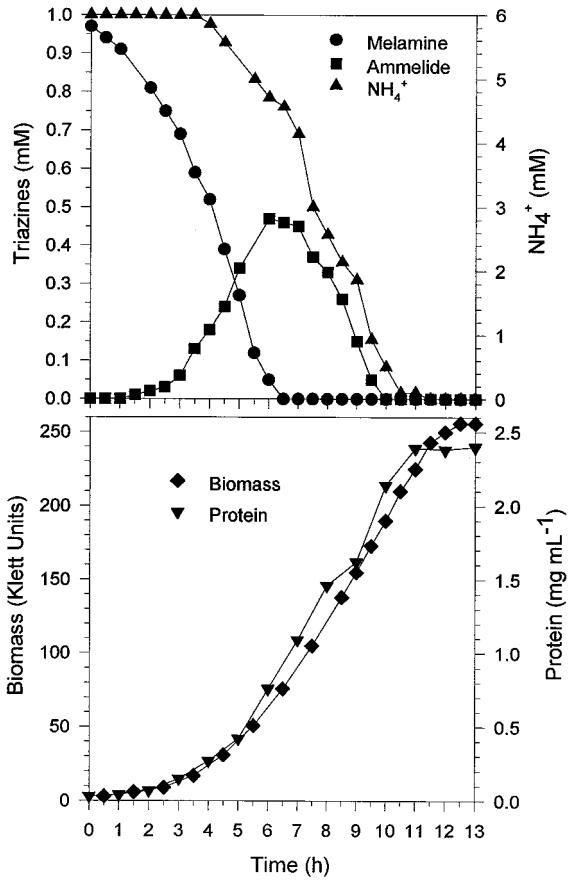

FIG. 3. Growth of *K. terragena* with melamine in the presence of 6 mM added NH_4^+. The upper panel shows the concentrations of melamine, ammelide, and NH_4^+; the lower panel shows turbidities and protein concentrations.

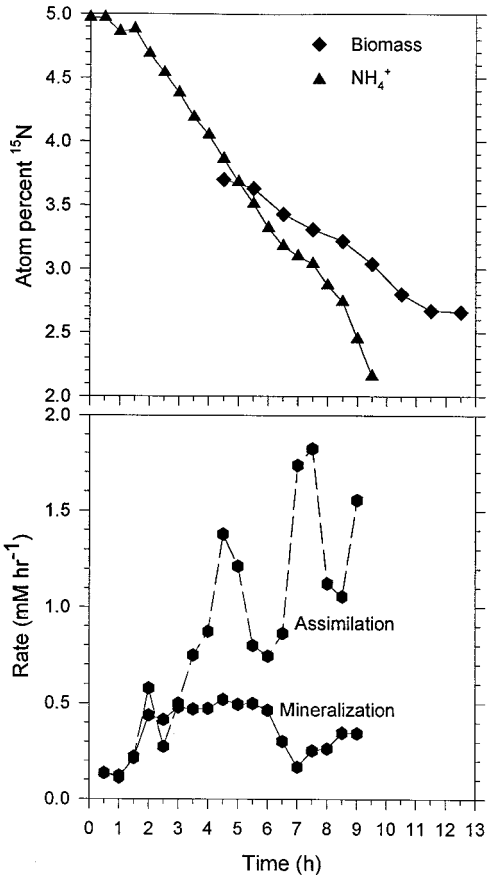

FIG. 4. Fate of N during growth of *K. terragena* with melamine in the presence of 6 mM added NH_4^+ (enriched with 5% ^{15}N). The upper panel shows changes in atoms percent ^{15}N of the external NH_4^+ pool and biomass; the lower panel shows rates of mineralization (triazine-N only) and assimilation (including both added NH_4^+ and triazine-derived NH_4^+). See Materials and Methods for details.

exhibited two major fluctuations at 5.5 to 6.5 h and 8 to 8.5 h (Fig. 4).

Rates of incorporation of N into biomass were more rapid from the pool of added NH_4^+ than from the triazine-derived N (Fig. 5). Nitrogen mass balances (biomass N plus triazine-N plus NH_4^+-N) could account for 11.1 ± 0.3 mmol (mean ± standard deviation) of total N between h 4.5 and 10.5; the final biomass N content was 12.07 mmol of N liter of culture^{-1}.

DISCUSSION

Turbidity and protein data are consistent with previously observed copious glycocalyx (slime) production by DRS-1 on solid agar medium. In both incubations (with or without added NH_4^+), there was a continued increase in turbidity after protein production had ceased, presumably due to the continued production of glycocalyx from carbon substrate. The observation that protein concentrations doubled when cells were cultured with 6 mM N (melamine only) versus 12 mM N (melamine + NH_4^+) indicates that nitrogen was the growth-limiting substrate in both incubations.

The transient accumulation of ammeline, ammelide, and cyanuric acid suggests that the primary mode of metabolism by DRS-1 is successive deamination of the triazine ring (Fig. 1). This pathway is consistent with studies by Jutzi et al. (8) on melamine metabolism by *Pseudomonas* sp. strain A. Although neither biuret nor urea was detected in the culture supernatant, presumably, cyanuric acid was hydrolyzed sequentially to yield biuret, urea, and finally ammonia. Based on nitrogen mass balances (^{15}N incubation), between 5 and 10% of triazine-N was unaccounted for during the growth phase, suggest-

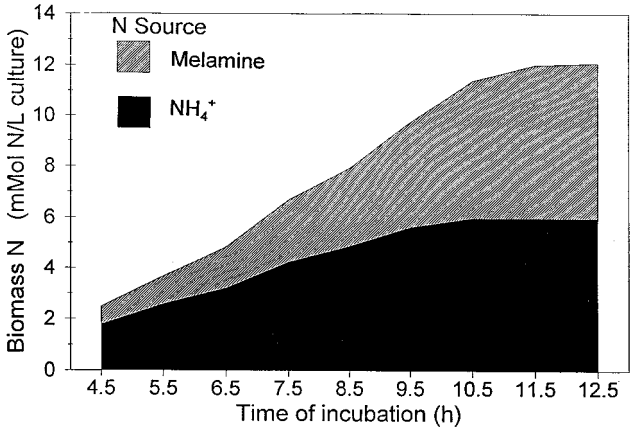

FIG. 5. Fate of N during growth of *K. terragena* with melamine in the presence of 6 mM added NH_4^+ (enriched with 5% ^{15}N). The assimilation of NH_4^+-N into biomass versus that of triazine-N is shown.

ing that biuret and/or urea accumulated transiently. Two independent observations indicate that triazine metabolism was essentially complete: (i) the twofold increase in protein production concomitant with a twofold increase in N (6 mM triazine-N plus 6 mM NH_4^+) and (ii) the close agreement between the measured final percent ^{15}N for biomass of 2.66 and the theoretical value of 2.68 [(5.0 + 0.366)/2].

Rapid melamine and ammeline metabolism in the presence of 6 mM NH_4^+ indicates that the initial steps of triazine metabolism were not inhibited by NH_4^+. The accumulation of ammelide in the supernatant of cultures grown with melamine and the lag in ammelide metabolism when present as the sole N source suggest that the regulation of the gene encoding ammelide aminohydrolase is independent of the regulation of melamine and ammeline aminohydrolase genes.

The decrease in atoms percent ^{15}N of the external NH_4^+ pool (Fig. 4) is indicative of dilution of the added NH_4^+ with triazine-derived NH_4^+, while the decrease in atoms percent ^{15}N of the biomass N reflects the assimilation of this external NH_4^+ pool during triazine-N mineralization. The calculated rates for mineralization (triazine-N) and assimilation (added NH_4^+ plus triazine derived NH_4^+) (Fig. 4) were based on changes in the external NH_4^+ concentration and the atoms percent ^{15}N of the external NH_4^+ pool. Integration of the rates of mineralization and assimilation provided estimates of the amount of triazine-N equilibrated with the external NH_4^+ pool as well as the total amount of NH_4^+ assimilated into biomass. Similarly, measurements of total N and atoms percent ^{15}N in biomass allowed independent estimates of triazine-N assimilation. Utilizing these two methods, the amount of equilibrated NH_4^+ assimilated between 4.5 and 9.5 h was ca. 6.8 mM, whereas the measured increased in biomass N during the same time period was 7.2 mmol liter of culture^{-1} (Fig. 5). These data indicate that between 4.5 and 9.5 h, and presumably up to 4.5 h, ca. 90% of the triazine-N was equilibrated with the external NH_4^+ pool prior to assimilation. Since only ca. 3.6 mM of triazine-N was mineralized by 9.5 h, no conclusions can be drawn regarding the fate of the remaining triazine-N.

In conjunction with the observations that (i) NH_4^+ accumulated in the culture supernatant concomitantly with melamine and ammeline metabolism (without added NH_4^+) and (ii) ammelide accumulated to ca. 40 to 50% (millimolar basis) of the initial melamine concentration (with or without added NH_4^+), the data suggest that the deaminations of melamine and ammeline, and possibly those of other intermediates, occurred outside the cytoplasmic membrane (i.e., in the periplasmic space and/or glycocalyx). This interpretation may be confounded, however, due to the potential for mixing of external and internal NH_4^+ pools from diffusion of NH_3 through the cytoplasmic membrane. Kleiner (10) has reported high rates of NH_3 exchange across the cytoplasmic membrane under physiological conditions under which the intracellular NH_4^+ concentration and pH are high relative to the extracellular NH_4^+ concentration and pH (setting up an NH_3 concentration gradient across the membrane), resulting in equilibration of internal and external NH_4^+ pools. It is unclear to what extent NH_3 exchange may have been a factor in these incubations.

The observation that rates of NH_4^+ assimilation fluctuated over the course of the incubation is intriguing. These data may suggest that NH_4^+ transport and/or assimilation were being transiently inhibited by some unknown intermediate(s). Further research will be required to elucidate this phenomenon.

The results of this investigation support the concept that *K. terragena* may be useful as an inoculant in conjunction with melamine as a fertilizer, due to the lack of NH_4^+ inhibition of melamine metabolism. However, several important questions, specifically, those of the viability of the organism in soil and the relative rates and extent of triazine metabolism under carbon-limiting conditions, remain.

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