

ANAEROBIC DEGRADATION OF CYANURIC ACID, CYSTEINE AND ATRAZINE

BY A FACULTATIVE ANAEROBIC BACTERIUM

by

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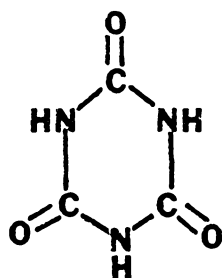
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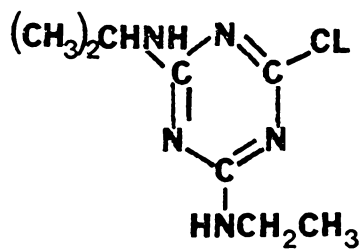
INTRODUCTION

The s-triazines comprise a large chemical family which has many uses in industry and agriculture. The relative recalcitrance of the triazine ring to biodegradation under aerobic conditions is well known. The chemical and physical properties of s-triazines was reviewed by Smolin and Rapaport (15) and the biodegradation of these compounds was reviewed by Esser et al. (5). At the time this study was initiated, no microorganism which could use the triazine ring as a carbon and energy source had been isolated, although the side chains of some highly substituted triazine compounds could serve as a carbon and energy source for some microorganisms (11). For example, F. F. Farmer and R. E. Benoit (unpublished data) tried, without success, to use long-term enrichments to isolate aerobic microorganisms, which could use simazine as a sole carbon and energy source. However, when [^{14}C] simazine was incubated in a mixed culture environment, where other energy sources were also available, degradation was observed (18). Recent studies have addressed the hypothesis that triazine compounds are decomposed as a result of cometabolism. Furthermore anaerobic conditions may provide a much more favorable environment than aerobic conditions for certain metabolic processes which favor triazine degradation. McCormick et al. (13) demonstrated that hexahydro-1,3,4-trinitro-1,3,5-triazine can be rapidly biodegraded in a complex medium using an activated sludge inoculum and anaerobic conditions, but not with aerobic conditions.

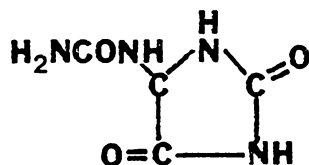
Since cyanuric acid (CA) is not chlorinated, or highly substituted (Fig. 1), it is one of the simplest models that can be used to study the biodegradation of the triazine ring. From a catabolic viewpoint, cyanuric acid may be an intermediate in the degradation of some complex s-triazine compounds, and, from an anabolic viewpoint, it may be an intermediate in nucleic acid biosynthesis. Evidence for the latter was largely based upon the observation that cyanuric acid was able to substitute for and support the growth of a uracil-requiring auxotrophic mutant of Escherchia coli (16). Several fungi that could aerobically degrade CA at a relatively slow rate were isolated by using CA as a nitrogen source, but not as an energy source (9) (18). Recently, the rapid degradation of [^{14}C] CA to $^{14}\text{CO}_2$ by Sporothrix schenckii was observed under aerobic shake culture conditions in media where carbohydrates served as the energy source. Furthermore, S. schenckii was able to utilize CA as the sole nitrogen source, and no CA intermediates were observed in the spent medium (19). Although there were no pure culture data to support the hypothesis that CA could be used as a microbial energy source, unequivocal data were available from several studies to demonstrate that CA was rapidly degraded in mixed culture systems. Wolf and Martin (18) observed that CA was completely degraded in soil, and Hauck and Stephensen (6) reported that nitrification of CA in anaerobic soil occurred after several months of incubation. The importance of aerobic conditions in the regulation of CA degradation was also observed by Saldick (12) who showed that sewage sludge degraded [^{14}C]CA under anaerobic, but not aerobic conditions. Wolf and Martin



Cyanuric Acid



Atrazine



Allantoin

Figure 1. Structures of Cyanuric Acid, Atrazine, and Allantoin

(18) obtained CA degradation in soil under anaerobic conditions, but found that aerobic conditions provided a more favorable environment for CA degradation in their system.

The first objective of this investigation was to isolate anaerobic bacteria that could degrade cyanuric acid in pure culture as a carbon and energy source. Therefore, enrichment cultures for the isolation of anaerobic bacteria that could degrade CA were initiated by inoculating anaerobic media in which CA was the sole carbon source with sediment from the holding pond of an industry which manufactured CA. The second objective of this study was to determine if these bacteria could degrade more complex triazine compounds, such as atrazine.

A preliminary report of this work was presented at the 80th annual meeting of the American Society for Microbiology. (Abstract of the annual meeting of the American Society for Microbiology, 1980, Miami Beach, Florida, p. 195)

MATERIALS AND METHODS

Anaerobic Enrichment Cultures and Bacterial Isolation. Stream sediment, which received effluent from an industrial holding pond in Charleston, West Virginia, was the inoculum for the enrichment cultures. CA was the principal organic compound in the waste water that had been added to this eutrophic pond for many years. Sediment samples were collected in polyethylene bags and kept at 0°C until processed within 24 h after collection. The CA-cysteine enrichment medium (CA-CYS medium) contained (g/l): CA, 1.5; cysteine HCl, 0.5; KH₂PO₄, 1.0; K₂HPO₄, 1.0; MgSO₄·7H₂O, 0.2; (NH₄)₂SO₄, 0.1; NaCl, 0.1; FeSO₄·7H₂O, 0.1; MnSO₄·7H₂O, 0.061; CaCl₂·2H₂O, 0.013; NaMoO₄·2H₂O, 0.00025; distilled water, 1000 ml. The pH of the medium was adjusted to 7.0 prior to autoclaving and resazurin (1 mg/l medium) was added as an oxidation/reduction indicator. All of the inorganic chemicals were reagent grade. CA (98% pure) and cysteine were obtained from Eastman Chemical Co. and J. T. Baker respectively. Other organic compounds were not detected in the CA preparation used throughout this study by use of the High Performance Liquid Chromatography or mass spectrograph assays. All anaerobic media were prepared as described in the VPI Anaerobe Lab Manual (8). Oxygen-free dinitrogen gas was used in the head space of all anaerobic media. All media were sterilized at 121°C for 15 min.

Enrichment cultures were initiated by placing 1 g of sediment in 10 ml of CA-CYS medium in anaerobic culture tubes. These cultures were

incubated at 30°C and transferred every two weeks before isolation of pure cultures was attempted. Pure cultures of bacteria were selected from roll tubes of CA-CYS medium with 1.5% agar (Difco) while using a dissection microscope. Multiple streaks on roll tubes were required to isolate pure cultures. A number of potential CA degraders were isolated based upon turbidity in the defined CA-CYS and CA-FeS media. The experiments described in this paper were conducted using the bacterial culture that had the highest turbidity and was therefore suspected to be capable of rapid degradation of CA and atrazine.

Defined CA-Mineral Salts Medium (CA-FeS medium). This medium was identical to the CA-CYS medium described above except FeS was used as a reducing agent instead of cysteine·HCl and 1 g of CA was used. A FeS suspension was prepared according to the method of Brock and O'Dea (4). FeS was added to the medium at a 1% concentration (vol/vol). Media were inoculated with 1 loop of culture in mid-log growth phase, grown in the CA-FeS medium and incubated at 30°C.

Analysis of Anaerobic CA Culture Media. It was not convenient to subsample a large flask of medium during the bacterial growth cycle, because of the possibility of oxygen contamination during the sampling procedure, which might effect the redox potential of the medium while flushing the system with oxygen free nitrogen gas. Therefore, multiple tubes of medium were inoculated for each experiment, and 6 culture tubes were selected at random for analysis at each sampling time. Duplicate samples were obtained by pooling the contents of 3 tubes. Combined subsamples were prepared anaerobically by placing 5 ml from

each culture tube in a sterile anaerobe tube flushed with sterile di-nitrogen gas. Viable cell counts were determined using triplicate roll tubes on brain heart infusion agar (Difco) of each anaerobic dilution taken from each subsample. Dilutions of the culture were made in brain heart infusion medium (Difco). Brain heart infusion medium was used rather than CA-CYS or CA-FeS media because the bacterium isolated in this study produced large colonies on the complex medium which expedited quantitative viable colony counts. The remaining spent medium was frozen and retained for chemical analysis. Samples were thawed in a 45°C water bath, and then passed through a 0.45 µm membrane filter (Millipore). Ammonia and cysteine were determined by the phenol-hypochlorine (1) and ninhydrin (7) methods respectively. CA was determined by the High Performance Liquid Chromatography (HPLC) method of Jessee et al. (10).

Analysis of Aerobic CA-CYS Culture Media. The anaerobic bacterium which degraded CA under anaerobic conditions in CA-CYS medium was placed in the same medium, but aerobic conditions were maintained throughout the experiment. The medium was prepared aerobically and dispensed in 250 ml quantities in plastic capped 500 ml Erylenmeyer flasks. One ml of a mid-log phase culture, which had been grown in the CA-CYS under anaerobic conditions, was used as the inoculum. The culture medium was incubated under shake-culture conditions at 30°C in a rotary bath incubator. The medium was sampled every 24 h. Cell counts were done using a standard dilution procedure on a brain heart infusion agar medium. In some experiments, CA or cysteine was eliminat-

ed from the CA-CYS medium in order to determine if either substrate could be used as a sole carbon source under aerobic conditions.

Atrazine Degradation in a Defined Medium under Anaerobic Culture Conditions. Atrazine (99.9% pure) was obtained from Ciba-Giegy, Greensboro, North Carolina. The culture medium (ATR-FeS medium) was identical to the CA-FeS medium described above, except atrazine (75 mg/l medium) was substituted for CA. The medium was prepared in 10 ml quantities in standard anaerobic culture tubes. The medium was sterilized at 121°C for 15 min. Each culture tube of ATR-FeS medium was inoculated with a mid-log growth phase bacterial culture which was grown in a CA-CYS medium. At each time interval after inoculation, 6 culture tubes were selected at random. The medium was analyzed for colony forming units and ammonia concentration using procedures similar to those described above to analyze the CA-FeS medium. Atrazine was determined by the method of Mattson (12). A Microtech 2000 gas chromatograph with a ^{63}Ni electron capture detector was used. The column, injector and detector temperatures were 175-185°C, 225°C and 335°C respectively.

RESULTS

Microbial Diversity of CA Degradation. A screen to isolate bacteria which could use CA as an energy source was initiated by selecting bacteria which had the greatest turbidity in a CA-CYS and CA-FeS enrichment media. The maximum turbidity observed in these anaerobic media was light, even in the most active cases, apparently because ammonia, or some other endproduct, or the lack of substrate prevented the production of high cell densities. No attempt was made to measure quantitatively the species diversity of CA decomposers isolated in this study, but based upon the preliminary morphological and biochemical data, there was a variety of anaerobic bacteria which could degrade CA. We restricted this study to anaerobic bacteria, but some microaerophilic bacteria were isolated which produced more turbidity than the anaerobic bacteria in the CA-CYS media when agar was added to achieve semi-solid conditions. We did not measure how much CA was degraded by these microaerophils in the spent media, but the potential of these organisms in triazine degradation experiments may be considerable. A sulfate reducing bacterium was isolated which was an active CA degrader, as shown by the HPLC assay, but this culture was not studied in detail.

The bacterium, that produced the greatest cell yield in the CA-CYS and CA-FeS media was selected for further study and is referred to in this work as CA bacterium. This gram negative rod shaped bacterium (1.3 x 0.75 μm) was a facultative anaerobe. It was capable of growth on trypticase soy and brain heart infusion media under aerobic and anaerobic conditions.

Growth of CA Bacterium on CA-CYS Medium. Cysteine was originally added to the enrichment medium because it helped to maintain a low redox potential in the defined medium. However, the use of cysteine in early phases of the enrichment procedure was responsible for the selective isolation of the CA bacterium which could degrade CA and cysteine (Fig 2). The maximum growth yield of 2.4×10^8 colony forming units/ml was observed after four days of exponential growth with little, if any, lag growth phase. During the first two days of the growth cycle, cysteine was the preferred substrate, and the CA was not metabolized. Rapid degradation of CA was initiated after the second day after the cysteine was utilized. The increase of ammonia in the medium was correlated with the degradation of CA. Cysteine can serve as a sole carbon and energy source for this bacterium, but only under anaerobic conditions. If CA was eliminated from the CA-CYS medium, a maximum cell yield of 1×10^6 colony forming units/ml was realized with cysteine as sole energy source. It is not known what role cysteine plays in the metabolism of this bacterium since other amino acids such as alanine could not serve as sole carbon and energy source. At the end of exponential growth phase, there was a short stationary phase followed by a decrease in culture viability presumably due to toxic concentrations of some end product such as ammonia, or lack of substrate necessary to maintain metabolism. The final pH of the medium was 7.5. There was no CA degradation in the uninoculated controls.

Growth of CA Bacterium on CA-FeS Medium. The degradation of CA in the medium where CA was the sole energy source is shown in Figure 3. The

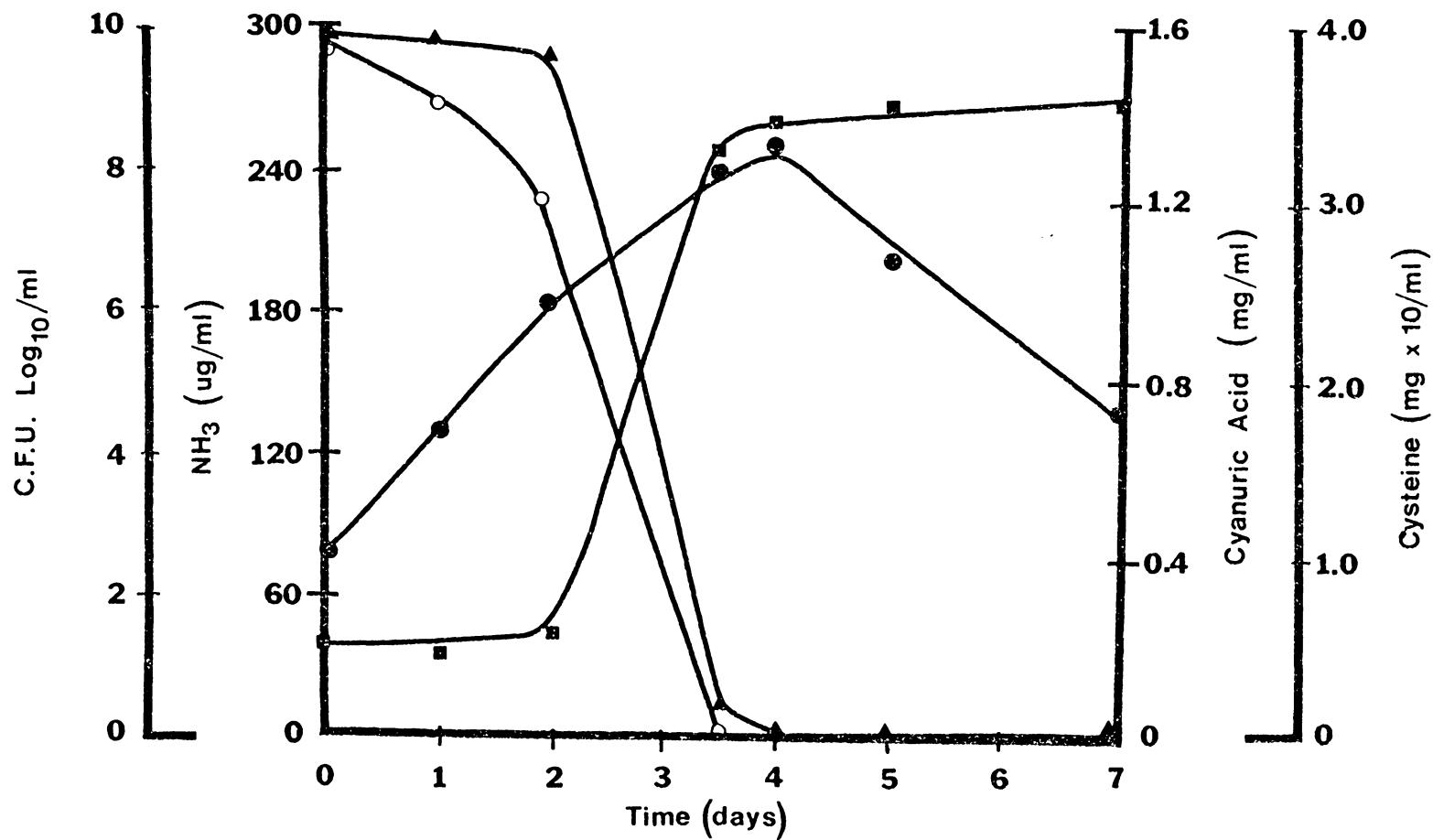


Figure 2. Anaerobic degradation of cyanuric acid by the CA bacterium in CA-CYS medium. Colony Forming Units; ●—●, Cyanuric Acid; ▲—▲, Cysteine; ○—○, NH₃; ■—■.

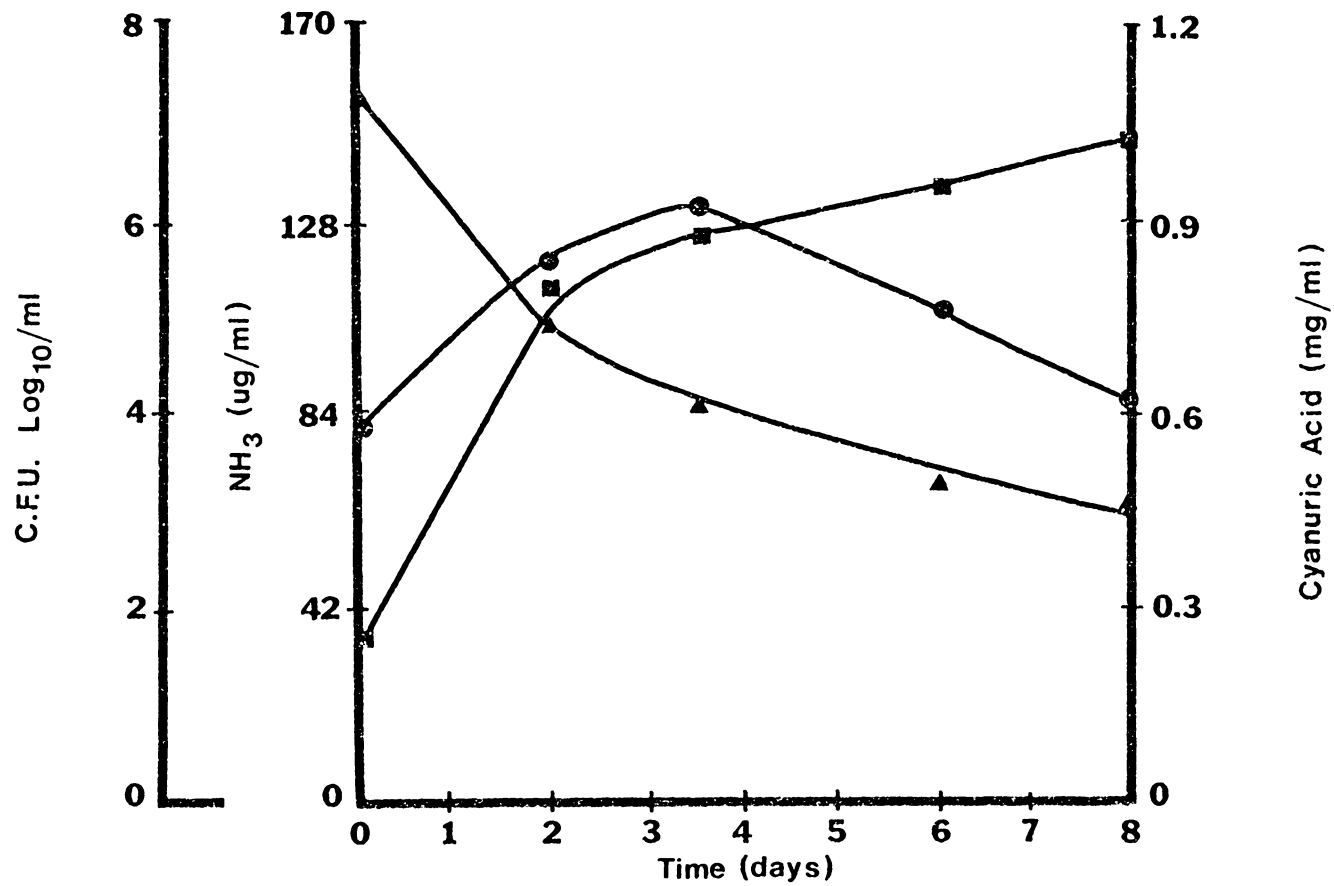


Figure 3. Anaerobic degradation of cyanuric acid by the CA bacterium in CA-FeS medium. Colony Forming Units: ●—●, Cyanuric Acid: ▲—▲, NH₃: ■—■.

growth of the culture was rapid during the first 2 days of the growth cycle. Stationary phase was reached 3 days after inoculation, and a maximum of 6.3×10^6 colony forming units/ml was observed. In most experiments, the amount of CA degraded was typical of the 640 ug/ml observed in this experiment after 8 days of incubation. However, in preliminary experiments, as much as 720 ug/ml of CA was degraded. In this experiment, growth ceased without using all of the substrate present. The amount of ammonia in the medium at the start of the growth cycle may have effected the quantity of CA degraded as well as the final ammonia concentration at the onset of the death phase. In both the CA-FeS and CA-CYS media, the CA bacterium had a very brief stationary phase followed by a rapid decrease in the number of viable cells in the death phase of the growth cycle. The final pH of the spent CA-FeS medium was 7.2. There was no degradation of CA in the uninoculated controls of this medium.

Growth of CA Bacterium on Atrazine-FeS Medium. The growth of the CA bacterium on the ATR-FeS medium is shown in Figure 4. Atrazine was degraded rapidly during the first day of the growth cycle with no apparent lag although the inoculum was grown on the CA-CYS medium. The maximum growth yield was 4.8×10^6 colony forming units/ml which was similar to the quantity of growth observed on the CA-FeS medium. Stationary phase was reached by the third day of incubation by this bacterium, therefore, the early phases of the growth cycle were similar for both the ATR-FeS media and CA-FeS media. However, the number of viable cells of the CA bacterium did not decrease sharply on the

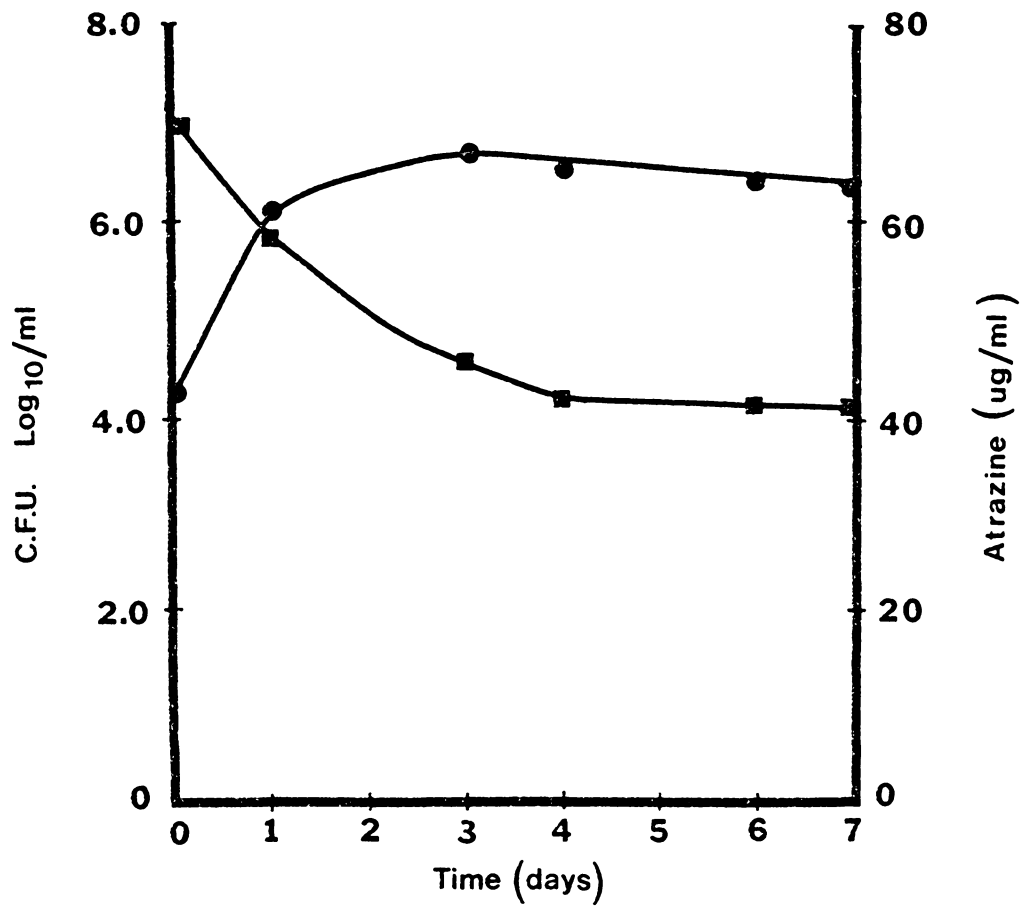


Figure 4. Anaerobic degradation of Atrazine by the CA bacterium. Colony Forming Units: ●—●, Atrazine: ■—■.

ATR-FeS medium as it did in the CA-FeS medium. The degradation of atrazine was correlated with the growth rate of the bacterium. Thirty ug/ml of atrazine was degraded during the 7 day incubation period. There was no degradation of atrazine in the uninoculated control media.

DISCUSSION

The facultatively anaerobic bacterium isolated in this study degraded CA under anaerobic, but not aerobic conditions in a defined medium. CA was the major carbon and energy source added to the defined medium. It will be necessary to perform ^{14}C labelled CA studies to prove if CA is meeting all the energy and carbon requirements of this bacterium. This is the first report of the isolation of a bacterium which can use CA as an energy source, although, Beilstein and Hutter (3) recently reported that a strain of Klebsiella pneumoniae could use CA as a sole nitrogen source. Since Saldick (14) observed biodegradation of CA under anaerobic conditions using activated sludge without an enrichment step, the genetic capacity to degrade CA may be widely distributed in many species of facultative, microaerophilic or strictly anaerobic bacteria in nature. The CA bacterium isolated in this work rapidly degraded CA, but in the CA-CYS medium the prolific growth of this bacterium was limited by the amount of substrate present. The ammonia present in medium and that produced when CA was degraded did not inhibit the rate of growth of the CA bacterium or the degradation of CA. Ammonia reduced the maximum growth rate of Klebsiella pneumoniae when it degraded CA, and CO_2 and NH_3 were the major end products. CA was degraded under aerobic and anaerobic conditions. Cell free extracts of Klebsiella pneumoniae were able to degrade CA under aerobic and anaerobic conditions. A model for CA degradation was proposed by Wolf and Martin (18). The pathway shown in this model may provide a mechanism whereby the CA bacterium can produce ATP by substrate phosphorylation

through the generation of carbamyl phosphate. Streptococcus allantoicus, which was isolated by Barker (2), was capable of using allantoin, oxamic acid and urea (in the presence of glyoxylate) as an energy source (17). S. allantoicus can form carbamyl phosphate from these three substrates. Since allantoin (Fig. 1) is closely related to cyanuric acid, the metabolic pathway used by the CA bacterium isolated in this work may be similar to that of S. allantoicus. Cell extracts of Klebsiella pneumoniae were able to degrade biuret which is an assumed intermediate in the degradation of CA. It is not known why this CA bacterium is unable to use CA as an energy source under aerobic conditions. The CA bacterium was able to use cysteine as a carbon and energy source. Cysteine was used only under anaerobic conditions. When cysteine was added to the defined CA medium, it stimulated the growth of the bacterium and increased the quantity of CA degraded. The CA was degraded after the cysteine was utilized with no apparent lag period since the log growth phase was exponential without a diauxic effect by CA bacterium in CA-CYS medium. This observation may have some important ecological implications; first, some substrates may inhibit the catabolism of CA in mixed culture systems (as a function of substrate concentration, abiotic conditions and ammonia concentration), and second, in some mixed culture systems CA degradation may be accelerated if other substrates increase the microbial biomass before CA degradation is initiated.

When cyclic compounds are subject to chlorine substitution or addition of some functional group on the ring carbon, frequently the

substituted derivative is more resistant to biodegradation than the original compound (11). The CA bacterium isolated in this study appeared to be an exception to this rule since atrazine was degraded as well as CA when they functioned as carbon and energy sources. It required only 30 ug/ml of atrazine to produce 4.8×10^6 colony forming units/ml of the CA bacterium, whereas, 640 ug/ml of CA was degraded in the production of 6.3×10^6 colony forming units by the same bacterium in the same medium and environment. Since the original inoculum used to start the enrichments came from an industrial source, it is possible that the microflora used to initiate this study were preselected for the capacity to degrade atrazine and CA.

No attempt was made to measure the species diversity of the bacteria in the enrichment cultures. The apparent diversity of CA degraders observed in the early phases of this study was important because those observations are consistent with the hypothesis that the potential to degrade CA is widespread in a broad spectrum of different bacterial species. However, these species may differ in their ability to use CA as a carbon, energy or nitrogen source. Since sulfate reducing bacteria are abundant in the sediment of many aquatic systems, it may be significant that a sulfate reducing bacterium that degraded CA was isolated in this study. However, until a quantitative study is made of the ecological distribution of CA degraders, these data should not be extrapolated to predict that CA degraders are widely distributed in nature. The isolation from the enrichment cultures of microaerophils, which produced higher cell densities than the CA bacterium used in

this study, may produce an interesting physiological model in addition to expanding the ecological niche where CA degradation is possible.

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Anaerobic Degradation of Cyanuric Acid, Cysteine, and Atrazine
by a Facultative Anaerobic Bacterium

by

Joel A. Jessee

(ABSTRACT)

A facultative anaerobic bacterium that rapidly degrades cyanuric acid (CA) was isolated from sediment of a stream that received industrial waste water effluent. CA decomposition was measured throughout the growth cycle by using a High Performance Liquid Chromatography assay while also measuring the concomitant production of ammonia. This bacterium used CA or cysteine as a major, if not sole, carbon and energy source under anaerobic, but not aerobic conditions in a defined medium. The cell yield was greatly enhanced by the simultaneous presence of cysteine and CA in the medium. Cysteine was preferentially used rather than CA early in the growth cycle, but all the CA was used without an apparent lag after the cysteine was metabolized. Atrazine was also degraded by this bacterium under anaerobic conditions in a defined medium.