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

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A facultative anaerobic bacterium that rapidly degrades cyanuric acid (CA) was isolated from the sediment of a stream that received industrial wastewater effluent. CA decomposition was measured throughout the growth cycle by using a high-performance liquid chromatography assay, and the concomitant production of ammonia was also measured. The bacterium used CA or cysteine as a major, if not the 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 of 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.

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 the s-triazines were reviewed by Smoline and Rapoport (15), and the biodegradation of these compounds was reviewed by Esser et al. (5). At the time our 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 carbon and energy sources for some microorganisms (13). For example, F. F. Farmer and R. E. Benoit (unpublished data) have tried unsuccessfully to use long-term enrichments to isolate aerobic microorganisms which can use simazine as the sole carbon and energy source. However, when [14C]simazine is incubated in a mixed-culture environment in which other energy sources are available, degradation is 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 do aerobic conditions for certain metabolic processes which favor triazine degradation. McCormick et al. (13) demonstrated that hexahydro-1,3,5-trinitro-1,3,5-triazine is rapidly biodegraded in a complex medium with an activated sludge inoculum under anaerobic, but not 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, CA 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 is largely based upon the observation that CA is able to substitute for and support the growth of a uracil-requiring auxotrophic mutant of Escherichia coli (16). Several fungi that can aerobically degrade CA at a relatively slow rate have been isolated by using CA as a nitrogen source but not as an energy source (9, 18). Recently, the rapid degradation of [14C]CA to 14CO2 by Sporothrix schenckii has been observed under aerobic shake-culture conditions in media in which carbohydrates serve as the energy source. Furthermore, S. schenckii is able to utilize CA as the sole nitrogen source, and no CA intermediates have been observed in the spent medium (19). Although there are no pure-culture data to support the hypothesis that CA can be used as a microbial energy source, unequivocal data from several studies demonstrate that CA is rapidly degraded in mixed-culture systems. Wolf and Martin (18) observed that CA is completely degraded in soil, and Hauck and Stephensen (6) reported that nitrification of CA in anaerobic soil occurs after several months of incubation. The importance of anaerobic conditions in the regulation of CA

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degradation was also observed by Saldick (14), who showed that sewage sludge degrades \([^{14}\text{C}]\)CA under anaerobic, but not aerobic, conditions. Wolf and Martin (18) obtained CA degradation in soil under anaerobic conditions but found that in their system, aerobic conditions provide a more favorable environment for CA degradation.

The first objective of this study was to isolate anaerobic bacteria that could degrade CA as a carbon and energy source in pure cultures. 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.


**MATERIALS AND METHODS**

**Anaerobic enrichment cultures and bacterial isolation.** Stream sediment which received effluent from an industrial holding pond in Charleston, W. V., was the inoculum for the enrichment cultures. CA was the principal organic compound in the wastewater 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 (CA-CYS) enrichment medium contained the following (grams per liter): CA, 1.5; cysteine-hydrochloride, 0.5; KH₂PO₄, 1.0; KH₂PO₄, 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; and distilled water, 1.000 ml. The pH of the medium was adjusted to 7.0 before the medium was autoclaved, and resazurin (1 mg/liter of 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 Kodak Co., Rochester, N.Y. and J. T. Baker Chemical Co., Phillipsburg, N.J., respectively. Other organic compounds in the CA preparation used throughout this study were not detected by use of the high-performance liquid chromatography (HPLC) or mass spectrophotograph analyses. All anaerobic media were prepared as described in the Virginia Polytechnic Institute and State University *Anaerobe Laboratory Manual* (8). Oxygen-free dinitrogen gas was used in the headspace 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 into anaerobic culture tubes. These cultures were incubated at 30°C and transferred every 2 weeks before isolation of pure cultures was attempted. Using a dissecting microscope, we selected pure cultures of bacteria from roll tubes of CA-CYS medium with 1.5% agar (Difco Laboratories, Detroit, Mich.). Multiple streaks on roll tubes were required to isolate pure cultures. A number of potential CA degraders were isolated on the basis of turbidity in the defined CA-CYS and CA-FeS (described below) media. The experiments were conducted with the bacterial culture that had the highest turbidity and was therefore suspected to be capable of rapid degradation of CA and atrazine.

**CA-FeS medium.** CA-FeS medium was identical to CA-CYS medium except that FeS was used as a reducing agent instead of cysteine-hydrochloride and 1 g of CA was used. An FeS suspension was prepared by the method of Brock and O'Dea (4). FeS was added to the medium at 1% (vol/vol). Media were inoculated with one loop of mid-log culture grown in CA-FeS medium and incubated at 30°C.

**Analysis of anaerobic CA culture media.** It was not convenient to sub sample a large flask of medium during the bacterial growth cycle because of the possibility of oxygen contamination during the sampling procedure, which might affect the redox potential of the medium while the system was flushed with oxygen-free nitrogen gas. Therefore, multiple tubes of medium were inoculated for each experiment, and six culture tubes were selected at random for analysis at each sampling time. Duplicate samples were obtained by pooling the contents of three tubes. Combined subsamples were prepared anaerobically by placing 5 ml from each culture tube into a sterile anaerobe tube flushed with sterile dinitrogen gas. Viable cell counts were determined on brain heart infusion agar (Difco)
with triplicate roll tubes 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 medium because large colonies of the bacterium isolated in this study formed 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 were passed through a 0.45-
μm membrane filter (Millipore Corp., Bedford, Mass.). Ammonia and cysteine were determined by the phenate (1) and ninhydrin (7) methods, respectively. CA was determined by the HPLC method of Jesse et al. (10).

Analysis of aerobic CA-CYS culture medium. 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 250-ml quantities were dispensed into plastic, capped, 500-ml Erlenmeyer flasks. One milliliter of a mid-log phase culture grown in CA-CYS medium 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 by a standard dilution procedure on a brain heart infusion agar medium. In some experiments, CA or cysteine was eliminated from the CA-CYS medium to determine if either substrate could be used as the sole carbon source under aerobic conditions.

Atrazine degradation in a defined medium under anaerobic culture conditions. Atrazine (99.9% pure) was obtained from CIBA-GEIGY Corp., Greensboro, N.C. The atrazine-FeS (ATR-FeS) culture medium was identical to CA-FeS medium except that atrazine (75 mg/liter of medium) was substituted for CA. We prepared 10-ml quantities of medium in standard anaerobic culture tubes and sterilized them at 121°C for 15 min. Each culture tube of ATR-FeS medium was inoculated with a mid-log bacterial culture which had been grown in CA-CYS medium. At each interval after inoculation, six culture tubes were selected at random. The medium was analyzed for colony-forming units (CFU) and ammonia concentration by procedures similar to those used to analyze CA-FeS medium (see above). Atrazine was determined by the method of Mattson et al. (12). A gas chromatograph (Microtech 2000) with a 63Ni electron capture detector was used. The column, injector, and detector temperatures were 175 to 185, 225, 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 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, some other end product, 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, but on the basis of preliminary morphological and biochemical data, we determined that there was a variety of anaerobic bacteria which could degrade CA. We restricted this study to anaerobic bacteria, but some microaerophilic bacteria isolated produced more turbidity than did the anaerobic bacteria in the CA-CYS medium when agar was added to achieve semisolid conditions. We did not measure how much CA was degraded by these microaerophilic bacteria in the spent media, but the potential of these organisms in triazine degradation experiments may be considerable. We isolated a sulfate-reducing bacterium which was an active CA degrader, as shown by the HPLC assay, but this bacterium 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 hereafter referred to as CA bacterium. This gram-negative rod (1.3 by 0.75 μm) was a facultative anaerobe. It was capable of growth on Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) 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 the 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 (2.4 × 10^8 CFU/ml) was observed after 4 days of exponential growth with little, if any, lag growth phase. During the first 2 days of the growth cycle, cysteine was the preferred substrate, and CA was not metabolized. Rapid degradation of CA was initiated at day 3 after the cysteine was utilized. The increase of ammonia in the medium correlated with the degradation of CA. Cysteine could serve as the 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 10^6 CFU/ml was realized with cysteine as the sole energy source. The role cysteine plays in the metabolism of this bacterium is not known, since other amino acids, such as alanine, could not serve as the sole carbon and energy source. At the end of the 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 the lack of substrate necessary to maintain metabolism. The final pH of the medium was

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FIG. 2. Anaerobic degradation of CA by CA bacterium in CA-CYS medium. Symbols: Ⓞ, CFU; ▲, CA; ○, cysteine; ■, NH₃.

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 in which CA was the sole energy source is shown in Fig. 3. The 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 × 10⁶ CFU/ml was observed. In this experiment, after 8 days of incubation, the amount of CA degraded was 640 μg/ml, similar to the amount we observed in other experiments. However, in preliminary experiments, as much as 720 μg of CA per ml was degraded. In this experiment, growth ceased without all of the substrate present being used. The amount of ammonia in the medium at the start of the growth cycle may have affected the quantity of CA degraded as well as the final ammonia concentration at the onset of the death phase. In both CA-FeS and CA-CYS media, 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 ATR-FeS medium.**
The growth of CA bacterium on ATR-FeS medium is shown in Fig. 4. Atrazine was degraded
rapidly during day 1 of the growth cycle with no apparent lag, although the inoculum had been grown on CA-CYS medium. The maximum growth yield was $4.8 \times 10^6$ CFU/ml, which was similar to the quantity of growth observed on CA-FeS medium. Stationary phase for this bacterium was reached by day 3 of incubation; therefore, the early phases of the growth cycle were similar for organisms grown on both ATR-FeS medium and CA-FeS medium. However, the number of viable CA bacterium cells did not decrease sharply on ATR-FeS medium, as was seen on CA-FeS medium. The degradation of atrazine correlated with the growth rate of the bacterium. Thirty micrograms of atrazine per ml 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 $[14C]$CA studies to prove that CA meets all of 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 can use CA as the 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 among many species of facultative anaerobic, microaerophilic, or strictly anaerobic bacteria in nature. CA bacterium 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 the medium and that produced when CA was degraded did not inhibit CA bacterium growth rate or degradation of CA. Ammonia reduces the maximum K. pneumoniae growth rate when this organism degrades CA, and CO$_2$ and NH$_3$ are the major end products under aerobic and anaerobic conditions (3). K. pneumoniae cell-free extracts were able to degrade CA under aerobic and anaerobic conditions. A model for CA degradation has been 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 allantonicus, which was isolated by Barker (2), is capable of using allantoin, oxamic acid, and urea (in the presence of glyoxylate) as energy sources (17). S. allantonicus can form carbamyl phosphate from these three substrates. Since allantoin (Fig. 1) is closely related to CA, the metabolic pathway used by the CA bacterium isolated in this work may be similar to that of S. allantonicus. K. pneumoniae cell-free extracts are able to degrade biuret (3), which is assumed to be an intermediate in the degradation of CA. It is not known why CA bacterium is unable to use CA as an energy source under aerobic conditions. CA bacterium was able to use cysteine as a carbon and energy source but only under anaerobic conditions. When cysteine was added to the defined CA medium, it stimulated the growth of CA bacterium and increased the quantity of CA degraded. The CA was degraded with no apparent lag after the cysteine was utilized, since the log growth phase was exponential without a diauxie effect 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, CA degradation may be accelerated in some mixed-culture systems 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 carbons, frequently the substituted derivative is more resistant to biodegradation than is the original compound (11). Atrazine was an exception to this rule in the case of CA bacterium: atrazine and CA were used as energy sources with equal facility. CA bacterium required only 30 $\mu$g of atrazine per ml to produce...
4.8 × 10^6 CFU/ml, whereas 640 μg of CA per ml was degraded in the production of 6.3 × 10^6 CFU/ml 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 among a broad spectrum of bacterial species. However, these species may differ in 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 microaerophilic bacteria which produced higher cell densities than did CA bacterium may produce an interesting physiological model, in addition to expanding the ecological niche where CA degradation is possible.

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LITERATURE CITED


