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Conversion of cyanide to formate and ammonia by a pseudomonad obtained from industrial wastewater

J.M. White, D.D. Jones, D. Huang and J.J. Gauthier

Biology Department, University of Alabama at Birmingham, Birmingham, AL, U.S.A.

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SUMMARY

A cyanide-degrading pseudomonad was isolated by selective enrichment in a chemostat inoculated with coke-plant activated sludge and maintained at a dilution rate of 0.042/h for 60 days with a feed of 10 mg/l cyanide. The isolate, a facultative methylotroph capable of growth on methanol and methylamine, degraded cyanide to formate and ammonia; it could utilize the released ammonia as a nitrogen source but did not further metabolize formate under the experimental conditions employed. Both cyanide-degrading enzyme activity and respiratory resistance to cyanide were inducible and were enhanced by repeated exposure to the compound. Cell-free extracts stoichiometrically converted cyanide to formate and ammonia in a reaction that did not require oxygen. Enzyme activity, lost upon dialysis, was restored by less than equimolar ratios of NAD(P)H or ascorbate to cyanide, indicating that the reductants did not function directly as co-enzymes.

INTRODUCTION

Cyanide is a highly toxic metabolic inhibitor that occurs in the wastewaters of industries involved in metal plating, pharmaceuticals, synthetic fibers, plastics, coal gasification, coal coking and ore leaching. These wastewaters must therefore be treated to remove cyanide prior to discharge into receiving waters. Since high concentrations of cyanide have been considered too toxic for direct biodegradation, most of the cyanide is removed by expensive chemical or physical means prior to biological treatment [5,12]. However, acclimatization of biological systems to high cyanide concentrations has been reported [5,11,15,17,19] and investigations in our laboratory have shown that chemostat cultures of steel industry activated sludge could, after a period of adaptation, treat coke-plant wastewater containing 200 mg/l of cyanide [7].

Studies of the microbiology of cyanide degradation have been hampered by the toxicity, volatility and reactivity of the compound, and the validity of reports of cyanide utilization as a sole source of carbon and nitrogen have been questioned [12]. It has been recognized, however, that investigating the utilization of cyanide with a continuous culture system would reduce the problem of toxicity [12].

Correspondence: J.J. Gauthier, Biology Department, University of Alabama at Birmingham, Birmingham, AL 35294, U.S.A.

Biological conversion of cyanide to formamide [3], and to ammonia and an unidentified carbon product [18] has been demonstrated in fungi. It has been proposed that a similar enzymatic pathway should exist in bacteria [1,11,12]. The products of the reaction, either formaldehyde, formaldoxime, or formate and ammonia, could serve as growth substrates for a cyanide-degrading bacterium. Alternatively, the released compounds could be utilized by other organisms in a mixed culture [20].

In this study, a pseudomonad able to degrade cyanide to formate and ammonia was isolated from an inoculum of coke-plant activated sludge maintained in a chemostat fed only cyanide. Although the isolate could grow on the one-carbon compounds methanol and methylamine, it did not utilize cyanide as a carbon source. The released ammonia was readily utilized as a nitrogen source.

MATERIALS AND METHODS

Selection of cyanide-degrading microorganisms

Cyanide-degrading microorganisms were selected by continuous culturing with a feed containing cyanide as the sole source of carbon and nitrogen. A 500 ml chemostat was inoculated with 500 ml of mixed liquor (1500 mg suspended solids) from the aeration basin of an activated sludge process treating coke-plant wastewater containing 200 mg/l phenol and 5–10 mg/l cyanide. The basal medium fed to the chemostat consisted of 0.02 M potassium phosphate buffer, pH 7.2, amended with 10 ml/l of a trace salts solution containing, per 1000 ml deionized water: $FeSO_4 \cdot 7H_2O$, 300 mg; $MgCl_2 \cdot 4H_2O$, 180 mg; $Co(NO_3)_2 \cdot 6H_2O$, 130 mg; $CaCl_2 \cdot 2H_2O$, 40 mg; $ZnSO_4 \cdot 7H_2O$, 40 mg; MoO_3, 20 mg. The complete feed contained 10 mg/l cyanide as the sole source of carbon and nitrogen; the dilution rate was 0.042/h. To establish equilibrium conditions, cyanide concentrations in the feed remained unchanged until day 61; then concentrations were increased by the increments indicated in Table 1.

Isolation of cyanide-degrading bacterium

After 60 days of chemostat operation, samples were serially diluted and plated onto nutrient agar, phenol agar and cyanide agar. The phenol medium consisted of trace salts in buffer (as above) amended with 0.02% phenol, 0.02% $(NH_4)_2SO_4$ and 0.005% yeast extract whereas cyanide media contained 0.02% or 0.05% cyanide and 0.005% yeast extract. After 5–14 days of incubation at 30°C, the isolates obtained by restreaking colonies onto nutrient agar were inoculated into trace salts in buffer amended with 100 mg/l cyanide and incubated at 30°C for 10 days. To determine resistance to cyanide and viability, the cultures were again plated onto nutrient agar. Growth of colonies was considered evidence for cyanide resistance. Cyanide-free maintenance medium consisted of trace salts in buffer amended with 3 g/l sodium lactate and 0.5 g/l $(NH_4)_2SO_4$.

Characterization

Isolated bacteria were characterized by gram staining, and by tests for motility, and catalase and oxidase reactions. Substrate versatility was assessed by the method of Stanier et al. [20].

The isolate from a phenol agar plate that represented the colony type present in greatest numbers, isolate 3, was chosen for further study. This isolate was tested for production of fluorescent pigments, aromatic ring cleavage, presence of arginine dihydrolase and poly-β-hydroxybutyrate, growth at 4 and 41°C, nitrate reduction [16], growth on carbon-free agar, growth on nitrogen-free lactate medium, and growth on single-carbon substrates.

Adaptation to cyanide and its degradation in batch culture

Pure cultures of isolate 3 were grown in 50 ml lactate medium contained in 300 ml sidearm flasks (Bellco Glass, Inc.) agitated at 200 rpm at 30°C. When exponential growth was established, as determined by measuring optical density with a green filter in a Klett-Summerson colorimeter (Klett Manufacturing Co., New York), cyanide was added at the concentrations indicated for each experiment. Cells able to grow in the highest concentration of cyanide tested were used as inoculum. For the second and subsequent passages, cyanide was added to the growth medium before inoculation to a cell density of $1–5 \times 10^7$ cells/ml.

Cell-free extracts

Adapted cells, grown to stationary phase in lactate medium containing 50 mg/l cyanide, were pelleted by centrifugation at 10000 × *g* for 10 min, concentrated five-fold, and resuspended in 0.02 M phosphate buffer (pH 7.2). The cells were disrupted (Cell Disruptor 350, Branson Sonic Power, Danbury, CT), and particulates were pelleted by centrifugation at 35000 × *g* for 30 min.

Analyses

Concentrations of cyanide and ammonia were analyzed with a Select-Ion 5000 ion analyzer (Beckman Instruments, Inc., Palo Alto, CA) equipped with specific ion electrodes (HNU Systems, New Highlands, MA). Concentrations of phenol were determined by optical density measurements at 260 nm (Beckman DU-8, Beckman Instruments, Inc., Palo Alto, CA). Oxygen consumption was measured with a biological oxygen monitor system (Model 53, Yellow Springs Instrument Co., Yellow Springs, OH). The protein content of cell-free extracts was estimated by Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA). Formate was assayed by the method of Hoepner and Knappe [10], and formamide by the method of Fry and Millar [3].

All ^{13}C-NMR experiments were performed at ambient temperature (23°C) on a Bruker WH-400 (9.4 Tesla) spectrometer operating at 100.62 MHz for ^{13}C. The chemical shifts of the ^{13}C resonances (broad band proton decoupled) were referenced to the external dioxane which was assigned a chemical shift of 66.5 ppm. The ^{1}H–^{13}C coupling constants were measured from the ^{1}H coupled spectra recorded with the decoupler gated off during data acquisition.

Cellular incorporation of cyanide-carbon was determined by adding ^{14}CN (NaCN, 9.0 mCi/mmol, New England Nuclear, Boston, MA) to a level of 100000 dpm/ml of unlabeled cyanide to the feed reservoir and to the reaction vessel. Protein was precipitated in 10% ice-cold trichloroacetic acid (TCA). Radioactivity in samples was determined using a liquid scintillation detector (Beckman LS 5801, Beckman Instruments, Inc., Palo Alto, CA).

Biomass determinations were based on optical density at 600 nm (Spectronic 20 spectrophotometer, Bausch & Lomb), microscopic cell counts, colony counts on nutrient agar, and cell dry weights after desiccation at 100°C.

RESULTS

Selection and characterization

A chemostat inoculated with coke-plant mixed liquor was fed 10 mg cyanide/l as the sole carbon and nitrogen source at a dilution rate of 0.042/h. During the first 30 days of operation viable cells declined from 1 × 10^9 per ml to approximately 1 × 10^7 per ml. After 60 days cyanide in the feed was increased incrementally (Table 1). At cyanide concentrations up to 30 mg/l the biomass remained constant and no cyanide was detected in the effluent. However, at 50 mg/l only 50% of the cyanide was degraded. A further increase of influent cyanide to 100 mg/l resulted in gradual washout of cells.

Serial dilution and plating onto cyanide agar yielded a few minute colonies that grew to the same size on agar plates lacking a carbon source. None of these isolates were able to degrade cyanide in liquid culture.

The predominant organism obtained by dilution plating on phenol agar was a gram-negative, motile, oxidase- and catalase-positive, rod-shaped bacterium. The isolate grew on glucose, glycollate, lactate, adipate, *p*-hydroxybenzoate, valine, tryptophan, glycerol, *β*-hydroxybutyrate and phenol, but did not grow on fucose, trehalose, cellobiose, maltose, starch, inositol, mannitol, geraniol, 2-ketoglutarate, maleate, parlagonate, testosterone, acetamide, arginine, norleucine, betaine or putrescine. No growth occurred when a source of carbon or nitrogen was omitted from the medium.

Good growth was obtained within 24 h on methanol (2 g/l) with ammonium sulfate as the nitrogen source. Growth occurred at a similar rate and with approximately the same yield on methanol plus cyanide (20 mg/l) after a 15 day lag period. Growth on methylamine as the carbon and nitrogen source occurred after a 5 day lag, whereas growth on methylamine plus cyanide occurred after an 8 day lag.

Table 1

Cyanide degradation by a continuous culture of microorganisms after 30 days of selection with cyanide

Culture volume: 500 ml; dilution rate: 0.042/h. During selection the culture received 10 mg cyanide/l.

Days	CN (mg/l)			Biomass[a] (mg dry wt.)		CN degradation[b] (mg/mg cell dry wt./h)
	feed	gas-trap	effluent			
31–60	10.0	0.2	0	1.2	1.2	0.17
61–67	30.0	3.0	0.2	1.2	1.5	0.42
68–77	50.0	23.3	1.8	1.5	1.5	0.35
78–82	100.0	53.8	5.2	1.5	0.8	0.75
83–90	200.0	58.0	101.2	0.8–>0		n.d.[c]

[a] Dry weight of biomass per 500 ml culture at beginning and end of the time increment indicated.

[b] Cyanide degradation was calculated by subtracting the cyanide in the gas trap and in the effluent from the cyanide in the feed, multiplying by the flow rate (500 ml × 0.042/h) and dividing by the average mg cell dry weight during the period.

[c] n.d. = not determined due to loss of biomass by washout.

No growth was detected on formamide, formate plus ammonium sulfate, urea or carbamate after 10 days of incubation. Growth on methanol and methylamine produced a pink pigment of a shade characteristic for each compound. No fluorescing pigment was produced on Garibaldi's medium [16]. Growth occurred at 41°C but not 4°C. Arginine dihydrolase was not produced and nitrate was not reduced. The organism performed meta-cleavage of protocatechuate.

Adaptation to cyanide

When cyanide was added to exponentially growing cultures, growth continued at a reduced rate for about 20 h (Fig. 1A), followed by lag periods of 50, 72 and 120 h in the presence of 5, 10 and 20 mg/l cyanide, respectively. After the lag periods growth recommenced at approximately the same rate as before cyanide addition, and the yields were equal to those of cyanide-free control cultures. During the second passage in cyanide, growth began after lag

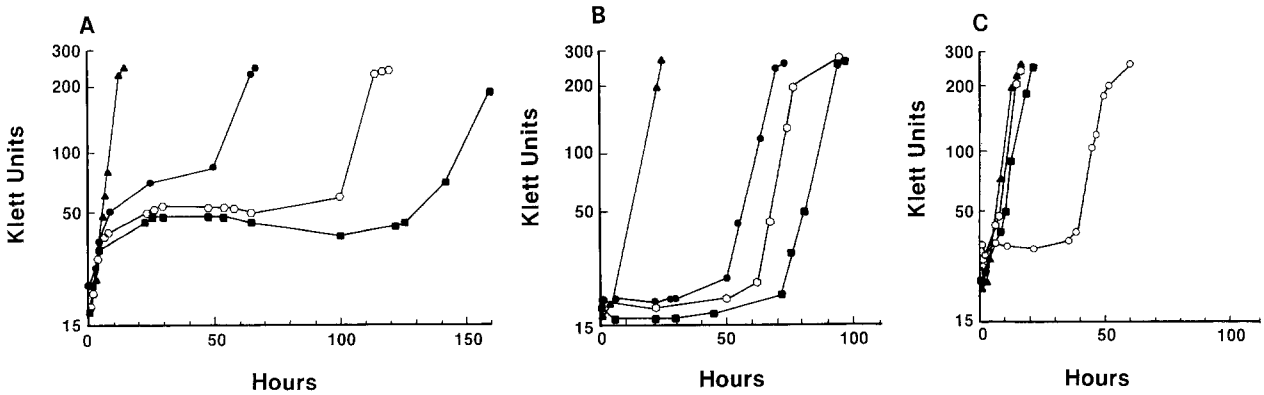

Fig. 1. Adaptation of isolate 3 to cyanide during the first (A), second (B) and third (C) passages in cyanide-containing media. Symbols for cyanide concentration, mg/l: (A) ▲, 0; ●, 5; ○, 10; ■, 20; (B) ▲, 0; ●, 5; ○, 10; ■, 20; (C) ▲, 0; ○, 10; ■, 20; ○, 50.

periods of 48, 62 and 75 h in media containing 5, 10 and 20 mg/l cyanide, respectively (Fig. 1B). During the third passage lag periods were further reduced to 4 and 5 h in the presence of 10 and 20 mg/l cyanide, respectively and to 38 h in 50 mg/l cyanide (Fig. 1C). During a fourth passage, significant lag periods, of 18 and 40 h, occurred only in the presence of 50 and 100 mg/l cyanide, respectively.

To determine whether adaptation to cyanide occurred in the absence of an added carbon source, unadapted cells were inoculated to a low density (0.1 mg cell dry wt./ml) into 20 mg/l cyanide in mineral salts–phosphate buffer. After an initial adaptation period of 72 h, cyanide was adjusted every 24 h to a final concentration of 20 mg/l. Four hours after each addition the cyanide concentration was reduced to less than 5 mg/l. The cells degraded four successive additions of cyanide at a steady rate of 0.52 (±0.03) μg/mg dry wt./min. The cell density remained constant.

Cells adapted by three passages in cyanide were used to assess the loss of adaptation to cyanide. Cultures were either grown to stationary phase and then left standing at room temperature for 24 h, or cells were inoculated twice in succession into cyanide-free lactate medium and grown to stationary phase. In media containing 10, 20 or 50 mg/l cyanide both cultures showed a similar loss of adaptation, reflected by lag periods of about 20, 40 and 85 h, respectively (Fig. 2), indicating that adaptation was gradually lost with or without growth. Repeated culturing on agar medium (five or six times) caused the complete loss of adaptation.

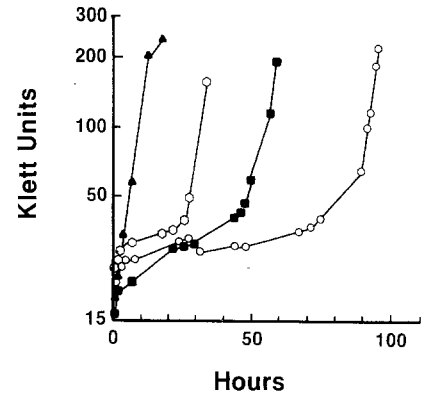

Fig. 2. Loss of adaptation of isolate 3 to cyanide after two successive passages in cyanide-free lactate minimal medium. Symbols for cyanide concentration in medium, mg/l: ▲, 0; ○, 10; ■, 20; ○, 50.

Growth of plated culture aliquots indicated that the cells remained viable during lag periods. Many rounded, pleomorphic, and incompletely dividing cells were observed during lag periods, but cells appeared normal after growth began.

Cyanide degradation in continuous culture

With lactate as the carbon source and cyanide the nitrogen source, up to 100 mg/l cyanide were degraded while cell dry weight increased more than six-fold (Table 2). With phenol as the carbon source, the cells were able to degrade only 50 mg/l cyanide (Table 3). An increase in cyanide to 100 mg/l resulted in a slow decline of the biomass and an increase in cyanide levels in the effluent. Analysis of effluent phenol indicated 95% utilization. With either lactate or phenol as the carbon source, am-

Table 2

Cyanide degradation by the isolate in continuous culture

Culture volume: 500 ml; dilution rate: 0.042/h. The feed contained 500 mg/l lactate; cyanide was the nitrogen source.

Days	CN (mg/l)			Biomass[a] (mg dry wt.)		CN degradation (mg/mg cell dry wt./h)
	feed	gas-trap	effluent			
1–7	50.0	3.8	0.2	10	24	0.056
8–18	100.0	8.0	0.2	24	64	0.044

[a] Dry weight of biomass per 500 ml culture at beginning and end of the time increment indicated.

Table 3

Cyanide degradation by the isolate in continuous culture

Culture volume: 500 ml; dilution rate: 0.042/h. The feed contained 250 mg/l phenol; cyanide was the nitrogen source.

Days	CN (mg/l)			Biomass[a] (mg dry wt.)		CN degradation (mg/mg cell dry wt./h)
	feed	gas-trap	effluent			
1–7	50.0	5.8	0.2	80	82	0.011
8–18	100.0	25.8	7.4	82	7	0.031

[a] Dry weight of biomass per 500 ml culture at beginning and end of the time increment indicated.

monia was produced in approximately stoichiometric amounts from the mineralized cyanide.

To assess cyanide degradation in the absence of an added carbon source, an inoculum of 100 mg dry weight of cells was fed cyanide at 250 mg/day. Although most of the cyanide was degraded initially, one third of the biomass was lost after 2 days and the experiment was terminated.

To simulate the low biomass levels of the original selection experiment, a chemostat was inoculated to a density of 4.5×10^7 cells/ml and fed 100 mg/l cyanide plus 2 mg/l yeast extract at the dilution rate of 0.017/h. For 21 days concentrations of cyanide and ammonia in the reactor fluid remained constant at 9.5 and 30 mg/l, respectively. The number of cells declined to 2.3×10^7/ml, indicating that the culture was growing at a rate somewhat slower than the chemostat dilution rate. However, TCA-precipitates of ^{14}CN-labeled samples contained only 0.1% of the radioactivity, not enough to indicate utilization of cyanide-carbon for biosynthesis. Simultaneously, radioactivity in the reactor fluid increased from the initial level of 1000 to 3000 dpm/ml. The accumulated carbon product was not carbon dioxide, since attempts to transfer the labeled compound to filters soaked in 20% KOH failed, even when samples were acidified.

Degradation of cyanide in batch culture

Approximately 33% of cyanide was lost from shaken, uninoculated media within 24 h due to air-stripping. During the first passage in cyanide, growth did not resume until the cyanide concentration had been reduced to 1–2 mg/l, and the rate of cyanide removal was the same as that due to air-stripping. During the second passage growth lagged until the cyanide concentration was reduced to 3–5 mg/l, but during this lag period measurable bacterial degradation of cyanide occurred. During subsequent passages, growth began in the presence of about 5 mg/l cyanide, and increased degradation rates were detected during lag periods. Normal growth rates were attained after lag periods of 18 and 40 h in cultures with 50 and 100 mg/l cyanide, respectively. The rate of degradation increased with increasing adaptation during subsequent passages in the presence of cyanide (Table 4).

Adapted, resting cells, concentrated to 4 mg dry wt./ml and resuspended in 100 mg/l cyanide in phosphate buffer, degraded from 0.2 to 0.5 μg cyanide/mg dry wt./min. Ammonia was released in equimolar ratios during cyanide degradation. Fully

Table 4

Cyanide degradation in lactate medium during lag periods before growth on cyanide

The cells were adapted to 20 mg cyanide/l.

No. of passages in CN^-	CN^- (mg/l)	CN^- degradation (mg/mg dry wt./h)
2	10.2	0.01
2	20.1	0.01
3	20.2	0.02
4	39.9	0.03
4	55.9	0.03

Table 5

The effect of cyanide on oxygen uptake by cell-free extracts

The endogenous rate of oxygen uptake by undialyzed extracts (3 ml) was determined for 5 min, then 0.2 ml of 38 mM cyanide (as KCN) was injected. The extracts were dialyzed for 5 h; the endogenous rate was determined, then 60 µl of 10 mM NADH or 90 µl of 10 mM ascorbate was injected; the rate of oxygen uptake was again determined and cyanide was injected (as above).

Cell-free extract	O_2 uptake (nmol/ml/min)			CN^- degraded in 60 min (µmol/ml)
	endogenous	with reductant	with CN^-	
Before dialysis	8.25	none used	3.75	2.4
After dialysis	3.38	4.31	0	1.5

adapted cells degraded two successive additions of cyanide without a loss of efficiency and a third addition at one-half the initial rate; a fourth addition was no longer degraded.

Cell-free extracts

Cell-free extracts degraded between 0.22 and 0.41 µg cyanide/mg protein/min. For each mole of cyanide degraded, 1 mol of ammonia and 1 mol of formate were released. The ^{13}C-NMR spectrum of $Na^{13}CN$ in 20 mM phosphate buffer showed a single resonance signal at 112.5 ppm. The degradation product did not show any signal at 112.5 ppm, but yielded a single resonance at 170.98 ppm. The proton coupled ^{13}C spectrum showed that this resonance is a doublet with a ^1H–^{13}C coupling constant (J_{CH}) of 195 Hz. The ^{13}C resonance of 100 mM sodium formate in 20 mM phosphate buffer has a chemical shift of 170.98 ppm and a coupling constant of 195 Hz. Identification was confirmed enzymatically by formate dehydrogenase assay.

Enzyme activity was retained in the frozen state but was lost after 24 h at room temperature. All activity was lost upon 5 h of dialysis against dilute phosphate buffer at 4°C. The addition of 0.2 mM NAD(P)H or 0.3 mM ascorbate restored enzyme activity sufficiently for the degradation of 1.5 mM cyanide (Table 5). Enzyme activity was fully restored in the presence of 1.5 mM NAD(P)H or 2.5% yeast extract. No activity was restored by the addition of mineral salts, 1% yeast extract or 1.5 mM NAD to the dialyzed extract.

Formamide (2.2 mM) was not degraded in either the presence or absence of cyanide.

Effect of cyanide on oxygen consumption

The endogenous rate of oxygen uptake by unadapted cells was depressed by about 25% after 10 min in the presence of 0.2 mg cyanide/mg cell dry wt., and substrate-stimulated oxygen uptake was inhibited by 90%. Oxygen uptake by adapted cells increased in the presence of cyanide (Table 6), and the substrate-stimulated rate was unaffected. These results suggest either non-specific stimulation of endogenous respiration or the direct involvement of oxygen in cyanide degradation. Since cyanide is degraded by cell-free extracts without oxygen uptake (Table 5), an oxygenase is not part of the enzyme

Table 6

Rate of oxygen uptake by resting cells adapted to 50 mg cyanide/l, in the presence of increasing concentrations of cyanide

Cell concentration: 4.5 mg/ml.

CN^- (mg/l)	nmol O_2/ml/min
0	8.3
50	16.7
100	15.0
200	12.0
300	9.6
400	9.0
500	1.5
700	0.5

system and there is no direct requirement for a reductant. The stoichiometry of degradation indicates that the overall reaction is:

$$HCN + H_2O \longrightarrow HCOOH + NH_3$$

DISCUSSION

The biological treatment plant from which the inoculum for enrichment of cyanide-degrading bacteria was taken routinely treated wastewater containing 200 mg/l phenol, 5 mg/l cyanide and 150 mg/l ammonia. The solids retention time was about 200 days and the hydraulic retention time was about 48 h. By feeding this population cyanide alone, an attempt was made to obtain organisms that would utilize cyanide as the sole source of carbon and nitrogen.

The inability to obtain colonies on cyanide agar is not conclusive evidence that organisms capable of growing on cyanide alone were not present. The toxicity of cyanide at high enough concentrations to yield visible colonies on plates and the reactivity of cyanide with ingredients in the agar medium could account for these results [12]. However, even though most of the cyanide fed to the chemostat was degraded when the cyanide concentration was as high as 30 mg/l, the lack of increase in biomass (Table 1) suggests that cyanide was not being used as a carbon source. The decrease in viable biomass in the chemostat from 10^9 to 10^7 cells/ml during the original selection period is also consistent with lack of growth on the cyanide carbon. A cyanide concentration of 30 mg/l should have provided about 7 mg carbon/day. At 50% efficiency, this should have yielded at least 3.5 mg of biomass in the chemostat. The 1.5 mg of biomass obtained could be accounted for by trace organic contaminants or cross-feeding of nutrients. For example, the formate produced by isolate 3 could have served as a carbon source for other organisms. It is unlikely that nitrifiers in the mixed culture contributed significantly to the biomass or to nutrient production, since most of the ammonia was recovered in the effluent. Finally, attempts to maintain growth of isolate 3 in a chemostat fed cyanide alone demonstrated retention of about 10^7 cells/ml, but no incorporation of cyanide carbon.

Isolate 3 grew on a variety of organic compounds, including phenol. It could utilize ammonia liberated from the cyanide as a nitrogen source but did not incorporate cyanide carbon, although one-carbon compounds such as methanol and methylamine served as growth substrates. Pseudomonads that facultatively utilize one-carbon compounds have been described [13,21]; to our knowledge, they have not been tested for the ability to degrade cyanide.

Utilization of methanol characterizes this isolate as a methylotroph. The accumulation of formate from cyanide degradation implies lack of formate dehydrogenase. Methylotrophs growing on methanol may possess an inducible formate dehydrogenase to oxidize formaldehyde [22]. This raises the question of whether formate dehydrogenase is present but suppressed in the presence of cyanide. This possibility will be considered in subsequent investigations.

Cyanide is very reactive and chemical reactions could form many compounds which the microbe could metabolize. However, many of these reactions take place slowly or under conditions differing from those found in culture media [1]. The kinetics and mass balance data reported here for growth in different media suggest that most of the cyanide was metabolized as cyanide.

Batch culture studies showed that the isolate adapted to increasing concentrations of cyanide. Although several passages in cyanide-containing media were necessary for cells to become fully adapted, growth was not a prerequisite for the initiation of adaptation. Unadapted cells suspended in phosphate buffer plus cyanide began to degrade cyanide after 72 h. Similarly, adaptation (both resistance and the ability to degrade) was lost in the absence of cyanide irrespective of cell growth. The pattern of adaptation during successive passages of growth in cyanide indicated the development of respiratory resistance [11,12,14] and the induction of a cyanide-degrading enzyme system that enabled the cells to detoxify the environment sufficiently for

growth to commence [8]. The adaptive nature of cyanide degradation may explain the instability of biological systems treating cyanide wastes.

The organism was able to degrade 100 mg/l cyanide efficiently in continuous culture when lactate was the carbon source but not when phenol was the carbon source. Since phenol was present in the effluent, the washout was not due to carbon limitation. A possible explanation for the ability of the organism to degrade 100 mg/l cyanide when lactate was the carbon source and the inability to degrade this same concentration in the presence of phenol is the combined inhibitory effect of two toxic substrates [6].

In the presence of cyanide the endogenous rate of respiration of unadapted cells was depressed, whereas that of adapted cells was stimulated. When concentrations of cyanide were higher than those to which cells were adapted, oxygen consumption declined gradually and then dropped sharply at a critical concentration when toxicity overwhelmed the respiratory system. Similar findings have been reported for other cyanide-degrading bacteria [4,8].

Harris and Knowles reported the isolation of a fluorescent pseudomonad that degraded cyanide to ammonia and carbon dioxide [8] in a reaction requiring NADH and oxygen. The intermediate product is thought to be cyanate [9]. Cyanide degradation by our isolate did not require oxygen. The endogenous rate of oxygen consumption of cell-free extracts actually fell to zero when cyanide was added to the reaction mixture. Although reductants were required to restore enzyme activity after dialysis of cell-free extracts, the low molar ratios of reductants to cyanide indicated that these compounds did not directly participate as co-factors in the reaction.

The ^{13}C chemical shift of the degradation product at 170.98 ppm suggests that the ^{13}C-labeled carbon is a carbonyl carbon. The doublet structure in the proton coupled spectrum further indicates that there is only one proton attached to the ^{13}C-labeled carbon. The resonance pattern and the ^{13}C chemical shift for the degradation product are in very good agreement with the NMR data on the 100 mM sodium formate in 20 mM phosphate buffer (σ = 170.98 ppm, and J_{CH} = 195 Hz). This result is consistent with formate being one of the final products of cyanide degradation.

The simplest pathway for the detoxification of cyanide is the conversion of HCN to formate and ammonia, either directly or via formamide as the intermediate compound [11]. Several species of *Pseudomonas* are able to utilize amides for growth [2]. Hydrolytic release of ammonia from formamide by amidase would yield formic acid. Formation of formamide from cyanide might therefore be expected to yield formate, with utilization of the formate depending on the presence or absence of relevant catabolic pathways. Inability of cell-free extracts of our isolate to degrade formamide and inability of this isolate to grow on formate plus ammonium sulfate indicates the lack of such a degradative pathway. It therefore appears that cyanide may be converted directly to formate and ammonia.

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REFERENCES

1 Castric, P.A. 1981. The metabolism of hydrogen cyanide by bacteria. In: Cyanide in Biology (Vennesland, B., ed.), pp. 233–261, Academic Press, London.

2 Clarke, P.H. and N. Ornston. 1975. Metabolic pathways and regulation: II. In: Genetics and Biochemistry of *Pseudomonas* (Clarke, P.H. and M.H. Richmond, eds.), pp. 263–340, John Wiley and Sons, New York.

3 Fry, W.E. and R.L. Millar. 1972. Cyanide degradation by an enzyme from *Stemphylium loti*. Arch. Biochem. Biophys. 151: 468–474.

4 Furuki, M., T. Yamamoto, T. Shimura and S. Tachibana. 1972. Studies on the biological treatment of cyanide-containing waste. Ferment. Technol. 50: 298–304.

5 Gaudy, A.F., Jr., E.T. Gaudy, Y.J. Feng and G. Brueggemann. 1982. The treatment of cyanide waste by the extended aeration process. J. Water Pollut. Control Fed. 54: 153–164.

6 Gaudy, A.F., Jr., W. Lowe, A. Rozich and R. Colvin. 1988.

Practical methodology for predicting critical operating range of biological systems treating inhibitory substances. J. Water Pollut. Control Fed. 60: 77–85.

7 Harden, D., D.D. Jones and J.J. Gauthier. 1984. Adaptation of an industrial activated sludge process to the removal of cyanide., In: Proceedings of the 38th Annual Purdue Industrial Waste Conference, (Bell, J.M., ed.), pp. 298–298, Butterworth Publishers, Boston.

8 Harris, R. and C.J. Knowles. 1983. Isolation and growth of a *Pseudomonas* species that utilizes cyanide as a source of nitrogen. J. Gen. Microbiol. 129: 1005–1011.

9 Harris, R.E. and C.J. Knowles. 1983. The conversion of cyanide to ammonia by extracts of a strain of *Pseudomonas fluorescens* that utilizes cyanide as a source of nitrogen for growth. FEMS Microbiol. Lett. 20: 337–341.

10 Hoepner, T. and J. Knappe. 1974. Determinations with formate dehydrogenase. In: Methods of Enzymatic Analysis, 2nd English Edn., Vol. 3 (Bergmeyer, H.U., ed.), pp. 1551–1555, Academic Press, Inc., New York.

11 Knowles, C.J. 1976. Microorganisms and cyanide. Bacteriol. Rev. 40: 652–680.

12 Knowles, C.J. and A.W. Bunch. 1986. Microbial cyanide metabolism. Adv. Microb. Physiol. 27: 73–111.

13 Mateles, R.I. and I. Goldberg. 1975. Utilization of C_1-compounds by *Pseudomonas* C. In: Microbial Growth on C_1-Compounds, pp.- 67–71, The Society of Fermentation Technology, Japan.

14 McFeters, G.A., D.F. Wilson and G.A. Strobel. 1970. Cytochromes in a cyanide-resistant strain of *Bacillus cereus*. Can. J. Microbiol. 16: 1221–1226.

15 Mudder, T.I. and J.L. Whitlock. 1984. Biological treatment of cyanidation wastewaters. In: Proceedings of the 38th Annual Purdue Industrial Waste Conference (Bell, J.M., ed.), pp. 279–287, Butterworth Publishers, Boston.

16 Palleroni, N.J. 1984. Pseudomonadaceae. In: Bergey's Manual of Systematic Bacteriology, Vol. 2 (Krieg, N.R., ed.), pp. 141–213, Williams and Wilkins, Baltimore.

17 Raef, S.F., W.G. Characklis, M.A. Kessick and C.H. Ward. 1977. Fate of cyanide and related compounds in aerobic microbial systems. I. Chemical reaction with substrate and physical removal. Water Res. 11: 477–483.

18 Shimizu, T. and H. Taguchi. 1969. Microbial treatment of industrial wastes containing cyanide. IV. Purification and some properties of cyanide-degrading enzyme of *Fusarium solani*. J. Ferment. Technol. 47: 639–643.

19 Shivaraman, N. and N.M. Parhad. 1984. Biodegradation of cyanide in a continuously fed aerobic system. J. Environ. Biol. 5: 273–284.

20 Stanier, R.Y., N.J. Palleroni and M.J. Doudoroff. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. 4: 159–271.

21 Thatcher, R.C. and T.L. Weaver. 1976. Carbon-nitrogen cycling through microbial formamide metabolism. Science 192: 1234–1235.

22 Trotsenko, Y.A. 1975. Alternate pathways of methanol metabolism in procaryotes and eucaryotes. In: Microbial Growth on C_1-Compounds (Terui, G., ed.), pp. 179–182, The Society of Fermentation Technology, Japan.