Degradation of the Metal-Cyano Complex Tetracyanonickelate(II) by Cyanide-Utilizing Bacterial Isolates

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Ten bacterial isolates capable of growth on tetracyanonickelate(II) $\{K_2[Ni(CN)_4]\}$ (TCN) as the sole nitrogen source were isolated from soil, freshwater, and sewage sludge enrichments. Seven of the 10 were identified as pseudomonads, while the remaining 3 were classified as *Klebsiella* species. A detailed investigation of one isolate, *Pseudomonas putida* BCN3, revealed a rapid growth rate on TCN (generation time, 2 h), with substrate removal and growth occurring in parallel. In addition to TCN, all isolates were able to utilize KCN, although the latter was significantly more toxic; MICs ranged from 0.2 to 0.8 mM for KCN and ≥ 50 mM for TCN. While growth occurred over a wide range of TCN concentrations (0.25 to 16 mM), degradation was most substantial under growth-limiting conditions and did not occur when ammonia was present. In addition, cells grown on TCN were found to accumulate nickel cyanide $[Ni(CN)_2]$ as a major biodegradation product. The results show that bacteria capable of growth on TCN can readily be isolated and that degradation (i) appears to parallel the capacity for growth on KCN, (ii) does not occur in the presence of ammonia, and (iii) proceeds via the formation of $Ni(CN)_2$ as a biological metabolite.

Large quantities of cyanide originate as wastes in the metal-plating, steel-tempering, and mining industries (11, 21, 30). In addition to cyanide, these wastes may contain other contaminants, including heavy metals. Waste effluents from electroplating processes, for example, contain significant quantities of nickel, cadmium, and chromium (20–22, 28, 32). Similarly, wastewaters in the mining industry, in which cyanide serves as an ore extractant, are high in nickel, copper, zinc, and iron (18). Although free cyanide (CN^- or HCN) is generally considered to be readily degraded by microbes (11, 12, 28), much less is known about its decomposition in wastes contaminated with metals. Since cyanide is highly reactive, it will readily bind metals as a strong ligand to form complexes of variable stability and toxicity (2, 24, 26). Examples include the tetracyano complexes of divalent nickel, copper, and zinc $\{[M(CN)_4]^{2-}$, where $M^{2+} =$ metal$\}$, the well-known hexacyano complex of iron (both Fe^{2+} [ferrocyanide] and Fe^{3+} [ferricyanide]), and related derivatives of chromium. These types of compounds are expected to constitute a significant fraction of cyanide-related wastes, but their degradation by microorganisms has not generally been investigated. Pettet and Mills (22) reported that the cyanide content of wastes containing heavy metals was reduced by acclimated sludge, but the responsible organisms were not identified. Rollinson and co-workers (23) showed that tetracyanonickelate(II) $\{[Ni(CN)_4]^{2-}\}$ (TCN) and, to a lesser extent, tetracyanocuprate(II) $\{[Cu(CN)_4]^{2-}\}$ were able to serve as nitrogen sources for the growth of *Pseudomonas fluorescens* NCIMB 11764 isolated originally on KCN. While growth on free cyanide (KCN or NaCN) by bacteria has been demonstrated in several cases (7, 9, 25, 29, 31, 32), the utilization of cyanide complexes has not. Thus, except for *P. fluorescens* NCIMB 11764 (23), we are unaware of the description of other organisms having this ability. We now report that bacteria capable of utilizing TCN

can readily be isolated from nature and provide further information on the physiological conditions and biochemical mechanism of TCN degradation.

(A preliminary account of some of this work has been presented [J. Silva-Avalos and D. A. Kunz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, Q180, p. 360].)

MATERIALS AND METHODS

Enrichment protocol, growth media, and cultivation of cells. Cyanide-utilizing isolates were obtained from enrichment cultures supplied with 0.25 to 0.5 g of soil or sewage sludge or 0.5 ml of creek water in 50 ml of minimal medium (15) containing 20 mM glucose as the carbon source and 0.25 to 0.5 mM TCN as the sole nitrogen source. All samples were obtained in the Denton, Tex., area or surrounding communities from sites having no previous history of cyanide contamination. Cultures were incubated at 30°C with shaking for 4 to 6 days; serial transfers (1% [vol/vol] inocula) were made every 48 h. Cells were streaked eventually to minimal agar (2% [wt/vol], purified; Difco) plates with compositions similar to that of medium used for enrichments, and single colonies were purified twice before taxonomic species identification.

Cultivation of cells on complete medium was accomplished with the formulation described by Lennox (L agar) (17). All isolates were stored as turbid suspensions in 10% dimethyl sulfoxide at −80°C.

Taxonomic species identification. Organisms were identified on the basis of cell and colony morphology, Gram stain reaction, and other physiological and biochemical tests in accordance with standard procedures (3, 8, 14). Identification of *Pseudomonas* strains was further based on criteria established by Stanier et al. (27). Electron microscopic examination of bacteria was accomplished with the phosphotungstic acid negative staining procedure of Dawes (5).

Growth determinations and biodegradation assays. The growth responses of individual isolates to various substrates were routinely tested in liquid cultures with the minimal medium described above. In each case, glucose (20 mM) was provided as the carbon source and either TCN or KCN was

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supplied as the nitrogen source. Control experiments with ammonia [$(NH_4)_2SO_4$, 1 to 10 mM] serving as the nitrogen source or medium completely lacking nitrogen were also included. Tests were conducted in 8 ml of medium contained in either test tubes (18 by 150 mm) or serum-stoppered bottles (120 ml) (Supelco, Bellefonte, Pa.). The latter were used exclusively for KCN determinations to prevent cyanide stripping by air. Inocula were cultivated in the same medium supplied with 10 mM $(NH_4)_2SO_4$, the cells were washed at least twice in Na-K phosphate buffer (pH 7.0) before being resuspended in the same buffer, and the A_{540} was adjusted to 1.0. A 2% (vol/vol) inoculum was provided in fresh medium supplied with the various nitrogen substrates at different concentrations. All cultures were incubated with shaking at 30°C for 48 h, at which time samples were removed and placed in a 1-cm cuvette, and the A_{540}'s were recorded.

The growth kinetics and biodegradation of TCN were examined in 250-ml Erlenmeyer flasks supplied with 50 ml of minimal medium by use of inocula prepared as described above. At various intervals, samples were removed from cultures and the cell density was determined by absorbance measurements before determination of the TCN content. The latter was accomplished by centrifuging samples in a Microfuge (Savant model HSC-10000) for 1 min and analyzing supernatants spectrophometrically. TCN was quantitated from its aqueous molar extinction value (ε) at A_{267}.

The degradation of KCN by isolate BCN3 was monitored similarly, but in this case inocula were prepared by cultivating cells in 1 mM NH_4Cl for 24 h until all available nitrogen had been depleted (A_{540}, 0.7). A 10% inoculum was added to 1 liter of minimal medium contained in a 2-liter Erlenmeyer flask, with no special measures being taken other than plugging flasks with cotton plugs covered with aluminum foil to prevent cyanide stripping by air. The cyanide (CN^-) content of culture samples removed during growth was determined colorimetrically by the method of Lambert et al. (16).

Determination of MICs. The MICs of cyanide and related substrates were determined in a Tris-buffered minimal medium formulated like that described by Rollinson et al. (23). The components of the medium were as follows: Tris buffer (40 mM), glucose (10 mM), $MgSO_4$ (2.2 mM), K_2SO_4 (5 mM), $FeSO_4$ (0.04 mM), sodium glycerophosphate (10 mM), and $(NH_4)_2SO_4$ (25 mM) (serving as the principal nitrogen source). In all cases, twofold dilutions of the test inhibitor were made in water and added in equal proportions to the above-described medium. Experiments were conducted in test tubes or in serum-stoppered bottles as described earlier and containing 8 ml of medium. A 2% inoculum previously grown on the same medium without inhibitor was added, and cultures were incubated for 48 h before being scored for growth by visible inspection or absorbance measurements. The MIC was defined as the lowest concentration of inhibitor above which no growth was observed.

Materials and analytical methods. KCN (99 atom%) was obtained from Aldrich Chemical Co. (Milwaukee, Wis.). TCN was prepared essentially as described by Fernelius and Burbage (6) by adding 24.8 g of $Ni(CN)_2$ (ICN Chemicals, Plainview, N.Y.) to an aqueous solution containing 29.2 g of KCN (Aldrich) in 30 to 40 ml of water. A red-orange solution was formed; when heated on a hot plate and allowed to cool, this solution yielded an orange precipitate. This precipitate was redissolved with heating and filtered hot, and the solution was allowed to cool; large yellow crystals were formed. Successive crops were collected and dried in a vaccum oven at 100°C to remove any water of hydration, and

TABLE 1. Identification of bacterial isolates obtained from TCN enrichments

Classification	Isolate designation	Enrichment source[a]
Pseudomonas putida	BCN3	Sewage sludge
Pseudomonas pickettii	BCN6	Soil
Klebsiella pneumoniae	BCN10	Soil
Pseudomonas putida	BCN21	Soil
Pseudomonas putida	BCN28	Soil
Pseudomonas putida	BCN29	Creek water
Klebsiella sp.	BCN31	Creek water
Pseudomonas putida	BCN32	Creek water
Klebsiella pneumoniae	BCN33	Creek water
Pseudomonas putida	BCN34	Creek water

[a] From separate sites in Denton, Tex., or surrounding communities.

the identity of the preparation was confirmed by UV spectroscopy and Fourier-transform infrared (FT-IR) spectroscopy. The aqueous UV spectrum (see Fig. 1B) had absorption maxima at 267 and 286 nm; a molar extinction coefficient (ε) of 11,200 M^{-1} cm^{-1} was calculated at 267 nm. Analysis of the crystalline solid (10% [wt/wt]) in KBr by FT-IR spectroscopy revealed doublets at frequencies (τ) of 2123.2 and 2128.1 cm^{-1} (see Fig. 5). The spectral properties of TCN were found to be consistent with those reported previously (4, 13, 24). Its purity was estimated to be about 95% from absorbance measurements made with aqueous solutions. All UV and FT-IR spectra were recorded, respectively, on LKB Ultrospec II and Nicolet 20SXB spectrometers. Other chemicals were from commercial sources and were used without further purification.

RESULTS

Isolation and identification of new isolates. Visible growth was evident in all enrichments supplied with either 0.25 or 0.5 mM TCN as the sole nitrogen source within 48 h following inoculation with soil, water, or sewage sludge. Continued growth was observed following several serial transfers to fresh medium, from which pure cultures were eventually obtained. Ten isolates (Table 1) from separate enrichments were retained and subjected to taxonomic classification tests. Six of the 10 were identified as fluorescent pseudomonads on the basis of positive oxidase tests, motility, and the production of diffusible fluorescent pigment. All six (BCN3, BCN21, BCN28, BCN29, BCN32, and BCN34) were unable to grow at 41°C, were negative for denitrification and gelatin hydrolysis, but were able to utilize benzylamine. These properties, among others, are most consistent with those described for *P. putida* biotype A (14, 27). Examination of isolate BCN3 by electron microscopy revealed several polar flagella, further supporting the classification of this strain, used for further studies outlined below, as *P. putida*. One pseudomonad (BCN6) that resembled *P. aeruginosa* in that it grew at 41°C and was able to denitrify nitrate was isolated. However, it produced no phenazine pigment when cultivated on King A medium (27) and was unable to grow on either adipic acid or geraniol. Further examination showed that it produced a brown diffusible pigment on complex medium, was able to grow on D-xylose but not trehalose or sucrose, and had a single polar flagellum. These properties are most consistent with those described for *P. pickettii* (14). Finally, strain NCIMB 11764, having previously been isolated on KCN as an enrichment substrate (9), was included in all taxonomic tests for com-

TABLE 2. Utilization of and tolerance to cyanide-related substrates[a]

| Isolate designation | Growth yield (A_{540}) on: | | | | | MIC (mM) of: | | |
| | TCN | | | KCN[b] (0.25 mM) | N⁻ (control) | TCN | KCN | NiCl₂ |
	0.25 mM	1 mM	16 mM					
BCN3	0.68	1.40	0.89	0.24	0.08	50	0.4	1.6
BCN6	0.60	1.37	0.82	0.15	0.10	>50	0.8	1.6
BCN10	0.51	1.05	0.46	0.16	0.06	50	0.4	0.2
BCN21	0.58	1.17	0.70	0.28	0.08	>50	0.8	0.8
BCN28	0.47	1.20	0.58	0.27	0.09	50	0.2	0.2
BCN29	0.40	1.12	0.57	0.14	0.07	50	0.8	0.2
BCN31	0.50	0.84	1.20	0.23	0.08	>50	0.8	0.2
BCN32	0.47	1.18	0.57	0.26	0.10	>50	0.4	0.2
BCN33	0.38	1.12	0.45	0.20	0.06	50	0.4	3.1
BCN34	0.89	1.43	0.34	0.23	0.10	50	0.4	1.6
NCIMB 11764	0.52	1.23	0.57	0.17	0.04	50	0.4	1.6

[a] All values represent the mean of at least two separate determinants made after 48 h of incubation.
[b] All determinations with KCN were performed with serum-stoppered bottles to prevent cyanide air stripping.

parative purposes, and its earlier classification as *P. fluorescens* (biotype II) was confirmed.

The remaining three oxidase-negative isolates, namely, BCN10, BCN31, and BCN33, were found to be nonmotile and capable of growth at 41°C. All three isolates produced smooth mucoid colonies on MacConkey agar and were lactose positive. Further analysis by inoculation into Enterotube II tubes (Roche Diagnostics, Nutley, N.J.) resulted in positive tests for citrate utilization, lysine decarboxylase, and urea hydrolysis, but all three strains were negative for indole production. These properties parallel those described for the genus *Klebsiella* (14). BCN10 and BCN33 were subsequently further identified as *Klebsiella pneumoniae*.

Growth of enrichment isolates and tolerance to cyanide substrates. Table 2 shows the results obtained when the growth and tolerance of all enrichment isolates were tested on TCN and KCN. *P. fluorescens* NCIMB 11764, previously shown to grow on TCN (23), was included in all experiments for comparisons. All organisms grew readily on TCN as the sole nitrogen source, with the maximum cell density being dependent on the amount of TCN supplied. Table 2 shows, for example, that growth on 0.25 mM TCN was significantly lower than that on 1 mM TCN. Growth on TCN at concentrations as high as 32 mM (data not shown) was observed, but above 8 mM the cell densities of all organisms decreased. With the exception of isolate BCN31, all strains had slightly lower absorbance measurements when cultivated on 16 mM TCN than when cultivated on 1 mM TCN.

Growth on KCN provided as the sole nitrogen source was also tested. Table 2 shows that there was substantially less growth of all isolates on 0.25 mM KCN than on the equivalent concentration of TCN. No significant growth above 0.25 mM KCN was observed (data not shown). We hypothesized that aside from the fact that TCN provides four times the number of nitrogen equivalents as does KCN, the differences in growth responses to the two substrates might be attributable in part to the higher toxicity of KCN. MICs determined for the two compounds with ammonia serving as the principal nitrogen source indeed showed that KCN was significantly more toxic (0.2 to 0.8 mM for KCN versus ≥50 mM for TCN; Table 2). These values, as might be expected, were somewhat higher than the maximum concentrations tolerated when both compounds were supplied as sole nitrogen sources for growth. Since TCN contains nickel metal, which is also toxic (1), it seemed appropriate to determine the relative MICs for it as well. The toxicity of

nickel was tested with NiCl₂, which ionizes freely to Ni²⁺. Without exception, this compound was also significantly more toxic than was TCN for all isolates tested.

Dependence of bacterial growth on the utilization of cyanide-related substrates. Figure 1A shows the growth curve for one enrichment isolate, *P. putida* BCN3, cultivated on 0.25 mM TCN in batch cultures. This strain was chosen to study the kinetics of TCN utilization, since preliminary experiments revealed that it grew faster than did most other isolates. The generation time of BCN3 on 0.25 mM TCN was estimated to be 2 h (from semilogarithmic plots not shown). This time is only slightly faster than that observed for growth on (NH₄)₂SO₄ (generation time, 1.45 h; see Fig. 4). Culture supernatants analyzed for TCN revealed a rapid rate of substrate consumption (Fig. 1B), with growth occurring essentially in parallel. Similar kinetics were observed for *P. fluorescens* NCIMB 11764 cultivated on 0.25 mM TCN; however, its growth rate (generation time, 5 h) (data not shown) was less than half that observed for BCN3. No significant decrease in TCN concentrations in uninoculated controls was observed.

To gain some assurance that the moderate increases in cell densities on KCN for most isolates (Table 2) were indeed substrate dependent, an experiment in which cyanide consumption was monitored during the growth of BCN3 was performed. The transfer of an ammonia-depleted culture to a medium containing 0.25 mM KCN was accompanied by a lag period of about 5 h, during which no growth occurred (Fig. 2). During this period, however, significant amounts of cyanide were consumed; thereafter, growth, as evidenced by increases in the culture turbidity, was apparent. Figure 2 also shows the results obtained when additional cyanide pulses were made to the culture. In each instance, cyanide removal was paralleled by increases in cell densities (ca. 0.15 to 0.20 absorbance unit), although these occurred in an asynchronous fashion. Irrespective of this result, growth appears to have been cyanide dependent. Interestingly, the rates of cyanide consumption upon subsequent pulses were dramatically faster than that observed upon initial inoculation, suggesting that adaptation to cyanide had occurred. Finally, cyanide removal, apparently due to air stripping, was also observed in an uninoculated control, but at significantly slower rates than in inoculated cultures.

Physiological conditions favoring TCN utilization by *P. putida* BCN3. Although TCN supplied at 0.25 mM was completely consumed by isolate BCN3, it seemed appropri-
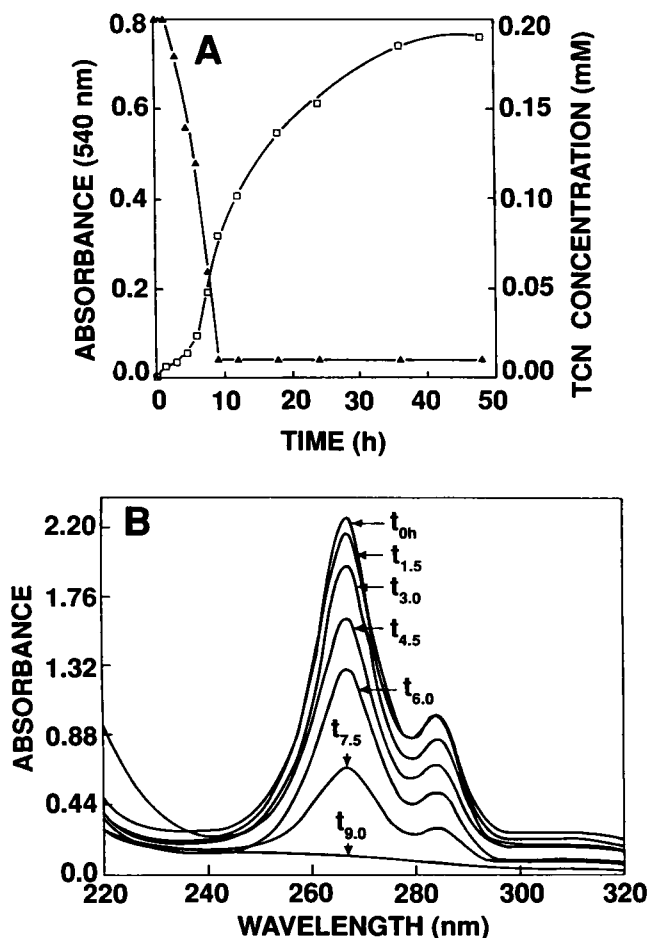
FIG. 1. Growth of *P. putida* BCN3 in a glucose (20 mM) minimal batch culture supplied with 0.20 mM TCN as the sole nitrogen source. (A) Growth (cell density [□]) and consumption of TCN (▲). (B) Spectrophotometric disappearance of TCN from culture supernatants analyzed at the indicated times (t) in hours.
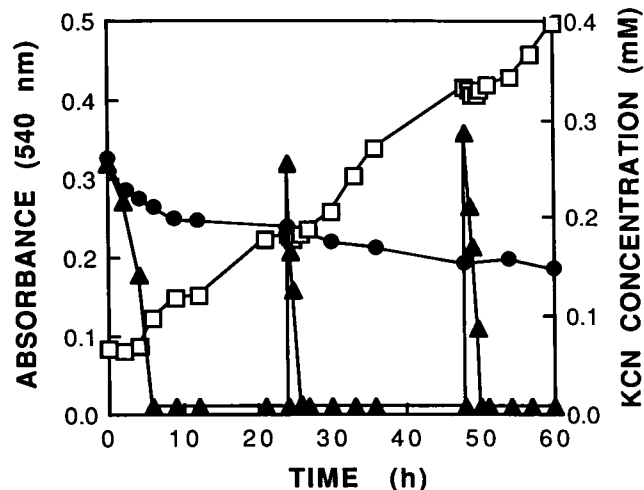

FIG. 2. Growth of *P. putida* BCN3 in a modified glucose (20 mM) minimal fed-batch culture (see Materials and Methods) pulsed at the times indicated with 0.25 mM KCN as the sole nitrogen source. Symbols: □, growth; ▲, KCN consumption; ●, disappearance of KCN in an uninoculated control.

ate to determine how much TCN was consumed at concentrations that might not be growth limiting. For example, while all organisms, including BCN3, grew readily at concentrations of TCN as high as 16 mM (Table 2), the extent of substrate utilization under these conditions was not known. Therefore, BCN3 was cultivated at various concentrations of TCN; following 48 h of incubation, the amount of substrate remaining in the medium was determined (Fig. 3). TCN was completely consumed when supplied at 0.25 to 1 mM; above this concentration, however, the extent of utilization decreased such that at 16 mM only 19 mol% (3.0 mM) was converted.

The effect of ammonia on TCN utilization was also tested, since it was hypothesized that ammonia might serve as a preferred growth substrate. Figure 4 shows a comparison between substrate utilization and growth of BCN3 cultivated on 1 mM TCN alone or in medium also supplemented with ammonia. When ammonia was present, TCN was not consumed.

Identification of nickel cyanide as a TCN biotransformation product. The cultivation of essentially all isolates on 8 to 16 mM TCN resulted in the formation of a flocculent green precipitate in the medium. This precipitate was not observed when cells were cultivated in the absence of TCN or in

non-TCN-inoculated controls, suggesting that its formation was growth dependent. To characterize this material, we centrifuged (5,000 × *g* for 10 min) the suspended solids from various cultures (e.g., BCN3, BCN6, BCN33, and NCIMB 11764), placed them in an oven at 60°C for 2 days, mixed the crude dry residue containing cell material and green solid with KBr (10% [wt/wt]), and analyzed the mixture by FT-IR spectroscopy. The major nickel-containing compound present in the crude solids from all four cultures was identified as nickel cyanide [$Ni(CN)_2$]. Figure 4 shows a comparison of the FT-IR spectra of commercial $Ni(CN)_2$, biological product, and TCN starting material. The absorption maxima for biological and authentic $Ni(CN)_2$ were identical (τ, 2172.4 cm^{-1}), and the spectra were distinguishable from the spectrum of unconsumed TCN remaining in the biological sample.

DISCUSSION

One of the main objectives of this research was to determine how readily organisms could be isolated from enrichment cultures supplied with TCN as the sole nitrogen source. These efforts resulted in the isolation of 10 organisms, 7 of which were identified as *Pseudomonas* strains and 3 of which were classified as *Klebsiella* strains (Table 1). We interpret the relative ease with which such organisms were isolated as an indication that TCN-degrading bacteria occur readily in nature. Although the full biodegradative potential of these isolates towards other cyano complexes has not been determined, their isolation parallels reports of the degradation of metal-containing cyanide wastes by sewage sludge (21, 22, 32) and other biological treatment processes (18). The isolation pf *Pseudomonas* strains is analogous to reports of the enrichment of similar organisms on KCN (9, 31) and to the description of *P. paucimobilis* as the principal agent in a biotreatment process for cyanide destruction in mining wastes (T. I. Mudder and J. L. Whitlock, U.S. Patent 4,461,834, 1984). On the other hand, the isolation of *Klebsiella* strains was somewhat surprising. To our knowledge there are no previous reports of cyanide utilization in this genus, but it is interesting that such organisms are generally
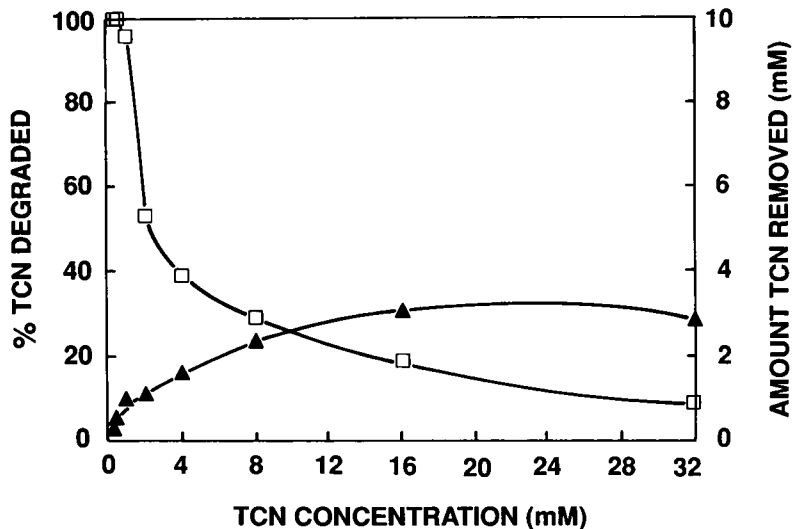
FIG. 3. Substrate consumption by separate cultures of *P. putida* BCN3 supplied with various concentrations of TCN as the sole nitrogen source for growth. Symbols: □, percentage of TCN degraded; ▲, amount of TCN consumed. All values represent the mean of at least two separate determinations.

considered to be cyanide resistant. Indeed, this property is often used as a taxonomic tool for differentiating this genus and other members of the family *Enterobacteriaceae* (14, 19).

In addition to growth on TCN, all 10 isolates appeared able to utilize KCN, although tolerance to the latter substrate was significantly lower. This result is reflected by the growth yields achieved at various substrate concentrations and in the MICs determined for the two compounds (Table 2). These findings are entirely consistent with the aqueous solution chemistry expected for the two compounds. For example, KCN readily disassociates in water, yielding toxic CN^- anions. In contrast, the CN^- ligands in TCN are tightly bound to the central nickel atom and are thus unavailable to

impart any toxicity; this fact is exemplified by the low disassociation (stability) constant of TCN (K_s [β_4], 10^{-22}) (2, 26). Thus, the differences in the growth response of bacteria and their tolerance to TCN and KCN can be attributed to substrate toxicity, which can be explained by the respective physical properties of the two compounds. The same explanation can be used to rationalize the reduced toxicity of nickel contained in TCN as compared with that of the free divalent metal (Ni^{2+}) (Table 2).

Detailed studies with isolate BCN3 showed that the growth of this organism and the removal of TCN from the culture medium were correlated (Fig. 1 and 4). The dependence of growth on TCN utilization is therefore inferred. It is noteworthy that at 0.25 mM TCN (Fig. 1), growth lagged
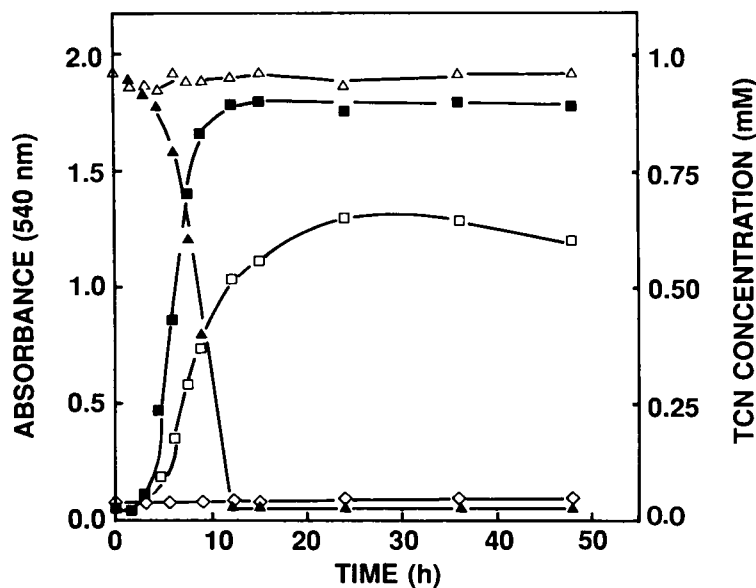

FIG. 4. Effect of added ammonia on the utilization of TCN as a source of nitrogen by *P. putida* BCN3. Shown are the growth (□ and ■) and consumption of TCN (▲ and △) in glucose (20 mM) minimal medium supplied with 1 mM TCN or in medium supplied with 1 mM TCN plus 10 mM $(NH_4)_2SO_4$. The growth of a control culture without added nitrogen is also shown (◇).
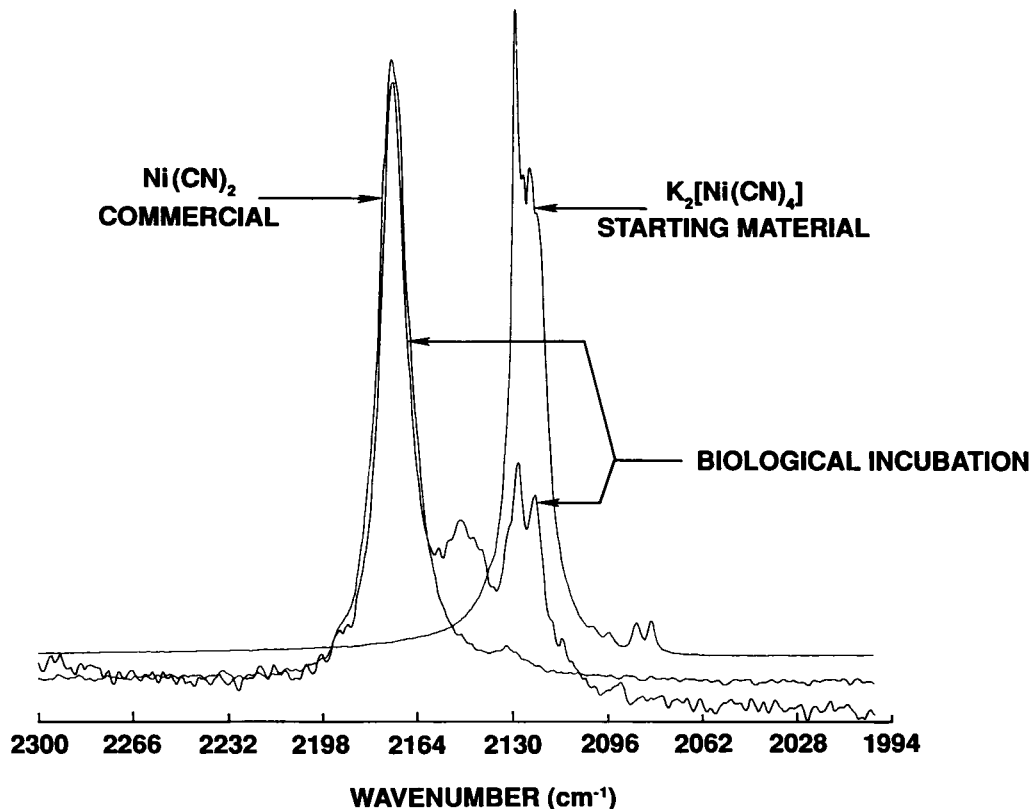
FIG. 5. FT-IR spectra of NiCN₂ biologically produced by *K. pneumoniae* BCN33 and authentic standard compounds. All spectra were recorded at an ambient temperature.

slightly behind substrate consumption (only ca. one-half the total growth yield was obtained at 100% substrate consumption). This result suggests that multiple events may be involved in TCN degradation, an idea that deserves further attention. Experiments in which KCN served as the growth substrate for BCN3 showed that even though growth was limited by substrate toxicity, increases in cell densities and substrate consumption were related (Fig. 2). It therefore appears that KCN is also capable of supporting the growth of this organism. Growth kinetics on KCN, observed in modified fed-batch cultures (Fig. 2), suggest that, as with growth kinetics on TCN, multiple events may be involved in the assimilation of KCN. The fact that KCN removal from the culture medium and growth occurred in a dramatically asynchronous fashion lends support to this idea. Moreover, it is of interest that the rates of cyanide removal by cells already adapted to cyanide appeared markedly faster than did those of unadapted cells. This finding suggests that cyanide degradation in this organism may be an inducible process, a finding consistent with earlier observations made with *P. fluorescens* NCIMB 11764 (9, 10). Since all other isolates in this study showed low but measurable growth responses to KCN (Table 2), it is reasonable to conclude that these organisms can also utilize this compound. These findings, taken together with the fact that *P. fluorescens* NCIMB 11764 could grow on TCN even though it was originally isolated on KCN (9, 23), suggest that, regardless of which compound is used for enrichment, the other appears to be equally well utilized. It is therefore tempting to speculate that the metabolism of the two compounds is related. While growth on KCN was limited by substrate

toxicity, growth on TCN occurred over a wide range of concentrations. However, degradation was most complete under growth-limiting conditions (0.25 to 1 mM TCN) (Fig. 3). These results indicate that the nitrogen content of the growth medium is important in somehow regulating TCN degradation. Experiments showing that degradation did not occur in the presence of ammonia (Fig. 4) are consistent with this conclusion and indicate further that the chemical form of available nutritional nitrogen is also important in regulating degradative attack.

Finally, the identification of nickel cyanide [Ni(CN)₂] (Fig. 5) accumulated by at least three separate isolates (including NCIMB 11764) strongly suggests that this compound is formed as a consequence of TCN degradation and represents a biological metabolite. Whether Ni(CN)₂ is further degraded is not presently known, nor is the mechanism by which it is formed. These are topics for future research. Previous investigations with strain NCIMB 11764 suggested that TCN degradation may involve a mechanism similar to that proposed for KCN degradation, involving an initial oxygenative attack generating ammonia, which can then be assimilated (10, 12, 23). However, it is unclear whether TCN is attacked directly by an oxygenase or whether CN⁻ is first released and then metabolized oxidatively to facilitate assimilation. Our observations that growth on TCN is accompanied by the accumulation of Ni(CN)₂ and that the ability to utilize TCN is paralleled by growth on KCN in all isolates tested could be taken as preliminary evidence that degradation proceeds via the latter mechanism. Further studies are needed to confirm this idea, however.

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