APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1992, p. 2022–2029 Vol. 58, No. 6
0099-2240/92/062022-08$02.00/0

Utilization of Cyanide as a Nitrogenous Substrate by *Pseudomonas fluorescens* NCIMB 11764: Evidence for Multiple Pathways of Metabolic Conversion

DANIEL A. KUNZ,* OLAGAPPAN NAGAPPAN, JUAN SILVA-AVALOS, AND GEORGE T. DELONG†

Department of Biological Sciences, University of North Texas, Denton, Texas 76203

Received 6 January 1992/Accepted 3 April 1992

The growth of *Pseudomonas fluorescens* NCIMB 11764 on cyanide as the sole nitrogen source was accomplished by use of a modified fed-batch cultivation procedure. Previous studies showing that cyanide metabolism in this organism is both an oxygen-dependent and an inducible process, with CO_2 and ammonia representing conversion products, were confirmed. However, washed cells (40 mg ml^{-1} [dry weight]) metabolized cyanide at concentrations far exceeding those previously described; 85% of 50 mM KCN was degraded in 6 h. In addition, two other C_1 metabolites were detected in incubation mixtures; their identities were confirmed as formamide and formate by ^{13}C nuclear magnetic resonance spectrocopy, high-pressure liquid chromatography, radioisotopic trapping experiments, and other analytical means. The relative yields of all four metabolites (CO_2, formamide, formate, and ammonia) were shown to be dependent on the KCN concentration and availability of oxygen; at 0.5 to 10 mM substrate, CO_2 was the major C_1 product, whereas at 20 and 50 mM substrate, formamide and formate were principally formed. The latter two metabolites also accumulated during prolonged anaerobic incubation, suggesting that *P. fluorescens* NCIMB 11764 can elaborate several pathways of cyanide conversion. One is formally similar to that proposed previously (R. E. Harris and C. J. Knowles, FEMS Microbiol. Lett. 20:337–341, 1983), involving the oxygen-dependent conversion of cyanide to CO_2 and ammonia. The other two, occurring in the presence or absence of oxygen, involve separate reactions to yield, respectively, formate plus ammonia or formamide. Since ammonia was detected essentially under all reaction conditions and no further evidence that formamide was further degraded was obtained, the utilization of cyanide as a provisional nitrogen source is presumed to proceed via ammonia as an assimilatory substrate.

A number of reports of the isolation of bacteria capable of growth on cyanide as the sole nitrogen source have appeared. Growth on both free cyanide (HCN or CN^-) (5, 8, 13, 14, 27–29) and metal-cyanide complexes {$[M(CN)_4]^{2-}$, where M^{2+} is a metal} (5, 22, 23) has been reported. Harris and Knowles (8, 10), for example, described the isolation of a number of cyanide-utilizing pseudomonads from soil, one of which, *Pseudomonas fluorescens* NCIMB 11764, converted KCN into products that included ammonia and carbon dioxide. The isolation of another cyanide-utilizing pseudomonad from a chemostat supplied with coke plant-activated sludge has also been reported (28). This organism, described as a methylotroph because of its ability to grow on methanol as a sole carbon source, also converted cyanide into ammonia, but in this case formate as opposed to CO_2 was identified as a metabolic by-product. Neither the latter organism nor strain NCIMB 11764 was capable of utilizing cyanide as the sole carbon and energy source, probably because at the concentrations needed for this purpose, cyanide is too toxic (8, 16). Once formed, ammonia is presumed to be assimilated by conventional pathways involving glutamine synthetase (EC 6.3.1.2)–glutamine:2-oxoglutarate aminotransferase (EC 1.4.1.13) and glutamate dehydrogenase (EC 1.4.1.3) enzymes (21, 26).

Although ammonia has been identified as a common end product of cyanide metabolism by several organisms (8, 10, 13, 28), the nature of the carbon-containing by-product and any potential conversion intermediates is less clear. On the basis of the following observations, Knowles and coworkers (9, 10, 15, 16) proposed that initial cyanide attack in *P. fluorescens* NCIMB 11764 was oxygenase mediated. First, washed cell suspensions and cell extracts did not catalyze the conversion of cyanide under anaerobic conditions. Second, cyanide turnover by cell-free preparations appeared to be correlated with simultaneous oxygen uptake and consumption of reduced pyridine nucleotide (NADH). Thus, the following conversion pathways leading ultimately to CO_2 and NH_3 and involving either monooxygenative or dioxygenative attack (equations 1 and 2, respectively) were proposed:

$$\mathrm{HCN} + \mathrm{O_2} \xrightarrow{\mathrm{NADH_2}\ \mathrm{NAD^+}} \mathrm{HOCN\ (cyanate)}$$
$$+ \mathrm{H_2O} \rightarrow \mathrm{CO_2} + \mathrm{NH_3} \quad (1)$$

$$\mathrm{HCN} + \mathrm{O_2} \xrightarrow{\mathrm{NADH_2}\ \mathrm{NAD^+}} \mathrm{CO_2} + \mathrm{NH_3} \quad (2)$$

It was further proposed that cyanate (cyanic acid), formed as a hypothetical reaction product of monooxygenation, was further metabolized by an enzyme resembling cyanase (EC 3.5.5.3), but further substantiation of this proposal has not proved possible (2, 10). Since unequivocal proof for either of these mechanisms of cyanide transformation by *P. fluo-*

* Corresponding author.
† Present address: Department of Chemistry, University of North Texas, Denton, TX 76203.

rescens NCIMB 11764 has not been forthcoming, additional studies were undertaken to better define the mechanism of cyanide transformation. The present work supports earlier research in which CO_2 and ammonia were identified as bioconversion products of cyanide metabolism. Further evidence is now also provided that formamide and formate represent additional metabolic products, implying that more than one pathway of cyanide transformation can be elaborated by this organism.

MATERIALS AND METHODS

Growth of cells. The source of *P. fluorescens* NCIMB 11764 and the minimal medium used for the cultivation of cells have previously been described (8, 17). Cells grown on KCN (1 to 2 liters) as the sole nitrogen source were cultivated by use of a modification of the fed-batch procedure described previously (23). The inoculum for growth was derived from a single colony taken from a 36-h-old nutrient (L-agar [23]) plate and cultivated in the batch mode (100 ml) on 1 mM NH_4Cl until all available nitrogen had been depleted (A_{540}, 0.7; 48 h). This culture was added (10% [vol/vol]) to a flask containing 1 liter of minimal medium supplied with glucose (20 mM) and KCN (0.25 mM), and the flask was incubated on a Gyrotory shaker at 30°C for 24 h before a second addition of 0.25 mM KCN was made. This procedure was repeated once more (total cultivation time, 72 h, with KCN additions totaling 0.75 mM; see Fig. 1) before cells were harvested by centrifugation and washed two times in Na-K phosphate buffer (pH 7.0). The cell pellet was resuspended in the same buffer at 200 mg ml^{-1} (wet weight) (A_{540}, 100; 40 mg ml^{-1} [dry weight]) and used for washed-cell bioconversion experiments or other purposes as described below.

Cyanide bioconversion by washed-cell suspensions. Bioconversion of cyanide by washed-cell suspensions was performed with sealed 15-ml serum vials (Hypo-Vials) (Pierce Chemical Co., Rockford, Ill.) to prevent volatile cyanide from escaping during the course of the incubation. Following the addition of cell suspension (0.5 ml; 40 mg ml^{-1} [dry weight]), the vials were crimp sealed with Tuf-Bond Teflon-rubber laminated discs (Pierce Chemical Co.). Reactions were initiated by the injection of KCN prepared as a fresh stock solution (0.1 to 1 M) in water immediately before use, and incubation mixtures were incubated at 30°C on a Gyrotory shaker (250 rpm). Samples were removed with a syringe at desired intervals and quickly centrifuged for 2 min in a microcentrifuge (Savant Instruments Inc., Farmingdale, N.Y.), and the supernatants were analyzed for cyanide and corresponding transformation products.

NMR spectroscopy. The analysis of ^{13}C-labelled conversion products by ^{13}C nuclear magnetic resonance (NMR) spectroscopy was conducted with washed-cell suspensions supplied with $K^{13}CN$ (99 atom%) at concentrations as high as 50 mM (3.26 mg ml^{-1}). The same general incubation procedure as that described above was used, and at various intervals following the addition of substrate, reaction mixture supernatants were transferred to NMR tubes. ^{13}C NMR spectra were recorded at an ambient temperature on either a Varian VXR-300 spectrometer at 75 MHz or a Varian GEM 200 instrument at 50 MHz. Chemical shift values for ^{13}C resonances (broad-band proton decoupled) were compared with benzene-d6 as an external standard, which was assigned a chemical shift value of 128.7 ppm (19, 25). Heteronuclear $J_{^{13}C\text{-}^{1}H}$ couplings were determined by acquiring data in the gated decoupled mode. Aqueous solutions of ^{13}C-enriched commercial compounds were prepared in Na-K phosphate buffer (pH 7.0) at concentrations ranging from 0.8 to 8.0 mg ml^{-1}, except for formamide, which was prepared at 65 mg ml^{-1}, and the resonance for naturally abundant ^{13}C recorded. The ^{13}C spectrum for bicarbonate was obtained either by gassing phosphate buffer with commercial $^{13}CO_2$ or by preparing a suspension containing $Ba^{13}CO_3$. At pH 7.0 these compounds equilibrate to yield $H^{13}CO_3^-$ as the principal species.

Analytical methods. Cyanide (as KCN) was measured colorimetrically by the method of Lambert et al. (18). Ammonia was determined by the indophenol method of Fawcett and Scott (4). A colorimetric method was also used to determine formamide on the basis of its conversion to the corresponding ferric hydroxamate (6, 24). For this purpose, 0.1 ml of sample was incubated for 10 min at 60°C with 0.2 ml of a 1:1 mixture of 3.5 N NaOH and 2.3 M hydroxylamine hydrochloride. To this mixture was added 0.1 ml of 4 N HCl and 0.1 ml of 1.23 M $FeCl_3$ prepared in 0.075 N HCl. The A_{540} was read 5 min after the addition of $FeCl_3$. Formate was determined enzymatically with commercial formate dehydrogenase (Sigma Chemical Co., St. Louis, Mo.) as described by Höpner and Knappe (12). Reaction mixtures contained the following in 0.4 ml: 18 to 20 μmol of KH_2PO_4 (pH 7.5), 0.4 μmol of NAD^+, 160 mU of formate dehydrogenase, and 0.01 to 0.02 ml (0.1 to 0.2 μmol) of biological sample. Reactions were initiated by the addition of formate dehydrogenase, and the change in the A_{340} was measured over 2 to 4 min. Since reactions proceeded only to about 62% completion, an average extinction value for NADH of 3.85 mM^{-1} cm^{-1} was routinely used to calculate the formate concentration. Spectrophotometic measurements were recorded on either an LKB Ultraspec II or a Perkin-Elmer Lambda 6 uv/vis instrument.

The detection and identification of formate and formamide in incubation mixture supernatants were further accomplished by high-pressure liquid chromatography (HPLC). Analysis was performed at an ambient temperature on an HPX-87H ion-exchange column (300 by 7.8 mm; Bio-Rad Laboratories, Richmond, Calif.) with a Rainin Dynamax system. The mobile phase consisted of 0.015 N H_2SO_4 containing 0.0004 M EDTA (pH 2.0) maintained isocratically at an elution rate of 0.5 ml min^{-1}. Compounds were detected by measuring the UV A_{210} with a Knauer variable-wavelength detector coupled to a MacIntosh computer by a Rainin HPLC data acquisition system.

Radiolabelling experiments. Radiolabelled metabolites were identified and recovered as either volatile (CO_2 and HCO_3^-) or nonvolatile (formamide and formate) products by use of $BaCl_2$ as a bicarbonate trapping reagent by the procedure of Fallon et al. (3). Reactions were initiated by the injection of 1 to 2.5 μCi of $K^{14}CN$ (47 mCi $mmol^{-1}$) into 1 ml of cell suspension contained in a 50-ml serum stoppered flask fitted with a center well. Nonisotopic KCN was also added to bring the substrate concentration to the desired level (0.5 to 50 mM). When the reactions were complete, as determined by simultaneous colorimetric assays, 0.3 ml of 4 N NaOH was injected into the center well to trap volatile CO_2 and the flask was incubated for an additional 15 min. At the end of this time, the contents of the center well and main compartment of the flask were removed and fractionated with $BaCl_2$ to recover volatile and nonvolatile products. For this purpose, 0.5 ml of the contents of the main compartment was centrifuged for 2 min in a microcentrifuge. Cell-associated radioactivity (cell pellet fraction) was determined after resuspending the pellet in 0.5 ml of phosphate buffer and

then adding this suspension to scintillation fluid. The supernatant (0.3 ml) was combined with 0.12 ml of 0.1 N NaOH plus 0.03 ml of 40% $BaCl_2$ for 5 min, and this mixture was separated into fractions designated main-compartment alkaline barium precipitate (containing bicarbonate) and main-compartment alkaline barium soluble (containing formamide plus formate) after centrifugation. After the pellet (alkaline barium precipitate) was washed with 0.2 ml of 0.12 N NaOH and the washing combined with the supernatant (alkaline barium soluble), the pellet was resuspended in 0.5 ml of 0.12 N NaOH and both it and the supernatant sample were added to scintillation fluid. The contents of the center well (0.3 ml) were treated in exactly the same fashion, generating fractions designated center-well alkaline barium precipitate (containing CO_2 and HCO_3^-) and center-well alkaline barium soluble (containing products other than CO_2 and HCO_3^-). All radioactive samples were added to 8 ml of Aquasol scintillation fluid (Dupont, NEN Research Products, Boston, Mass.) and counted on an LS 7000 scintillation counter (Beckman, Irvine, Calif.).

Metabolism of formamide. The metabolism of formamide was tested in several ways. First, cells were tested for the ability to grow on formamide as a sole nitrogen source. This was accomplished in glucose minimal medium with formamide supplied at concentrations ranging from 0.5 to 20 mM by cultivation procedures already described (23). Second, washed-cell suspensions were tested for the ability to degrade formamide after growth on cyanide. The procedures for conducting these experiments were similar to those already described with cyanide as a bioconversion substrate. Reactions were initiated by the addition of 0.5 to 20 mM formamide to washed cells, and the time-dependent disappearance of substrate was monitored colorimetrically. The third approach was to test for formamide degradation by cell extracts. Crude extracts were prepared as described elsewhere (17) and incubated with formamide at 30°C. Incubation mixtures contained 0.5 ml of crude extract (ca. 20 mg of protein ml^{-1} [20]), and reactions were initiated by the addition of 0.5 to 20 mM formamide (2.5 to 50 μl). In each case, simultaneous incubations with crude extract incubated in the absence of substrate were included as controls. Samples from each were taken at desired intervals and assayed directly for formamide, with the control sample being used as a reference for colorimetric determinations.

Chemicals. KCN (97%), formamide (≥99%) sodium formate (≥99%), formic acid (95 to 97%), $K^{13}CN$ (99 atom%), $Ba^{13}CO_3$ (98 atom%), ^{13}C-labelled Na formate (99 atom%), and $^{13}CO_2$ (99 atom%) were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). These and other chemicals from commercial sources were used without further purification.

RESULTS

Growth and cyanide consumption. A typical time course for fed-batch cultivation of NCIMB 11764 in minimal medium supplied with KCN as the sole nitrogen source is shown in Fig. 1. An initial lag period of about 10 h was observed after the addition of KCN, during which little growth occurred but most of the cyanide was consumed. After this, growth commenced until the culture appeared to become nitrogen limited (20 h), at which time KCN (ca. 0.25 mM) was again added. Although cells rapidly consumed the added KCN, a short lag period (ca. 5 h) was again observed before growth resumed. A similar relationship between substrate consumption and growth occurred when subsequent KCN additions were made (24 and 48 h), with the

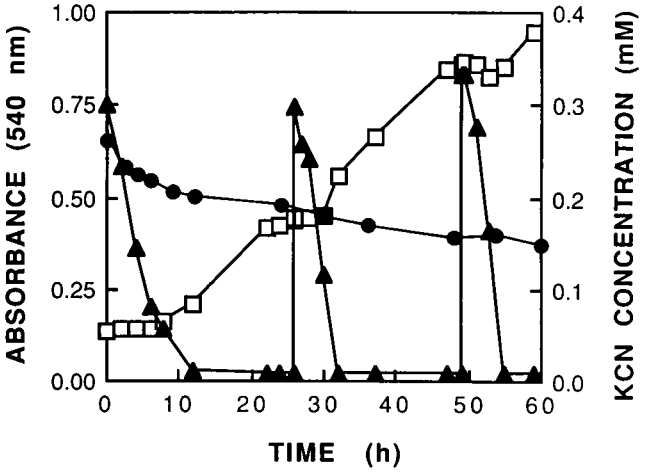

FIG. 1. Growth of *P. fluorescens* NCIMB 11764 in a modified glucose (20 mM) minimal medium fed-batch culture pulsed at the times indicated with 0.25 mM KCN as the sole nitrogen source. Symbols: □, growth; ▲, KCN consumption; ●, disappearance of KCN in an uninoculated control.

removal of KCN on successive additions proceeding at slightly faster rates over that seen initially. The generation time (calculated from semilogarithmic plots; data not shown) of cells cultivated in this manner was estimated to be 8 h. The final turbidity (A_{540}) achieved after 3 days of cultivation was approximately 0.8, equivalent to 0.32 mg of cell dry weight ml^{-1}. Little or no growth was observed when KCN was added to fed-batch cultures in excess of 0.25 mM.

Bioconversion of cyanide by washed-cell suspensions. Washed cells of NCIMB 11764 catalyzed the rapid time-dependent disappearance of KCN following growth on this compound as the sole nitrogen source. Initial experiments conducted at low substrate concentrations (0.25 to 1 mM) revealed that cyanide was rapidly consumed (ca. 2 nmol min^{-1} mg^{-1} of cell dry weight), but attempts to demonstrate the accumulation of ammonia under these conditions were unsuccessful. Further experiments showed that cells were capable of metabolizing cyanide over a broad range of KCN concentrations. For example, the degradative attack of KCN supplied at concentrations as high as 100 mM (6,510 ppm) was observed, and at 1 mM or above, the accumulation of ammonia could consistently be demonstrated. Typical reaction kinetics for a washed-cell suspension supplied with 10 mM KCN are shown in Fig. 2. The rapid removal of cyanide by KCN-grown cells (ca. 5 nmol min^{-1} mg^{-1} of cell dry weight) was accompanied by the concomitant formation of ammonia produced in about 40% molar yield. The results in Fig. 2 further show that when an analogous incubation mixture was made anaerobic by flushing with N_2, cyanide removal occurred at a significantly reduced rate. Similar results were observed regardless of the amount of KCN supplied in anaerobic incubations. Finally, in contrast to cells grown on KCN, ammonia-grown cells showed little or no cyanide bioconversion activity.

^{13}C NMR detection and identification of cyanide biotransformation products. The finding that cells of NCIMB 11764 were capable of transforming high concentrations of KCN greatly facilitated the detection and identification of additional reaction products besides ammonia. Since earlier reports (9, 10, 16) of cyanide metabolism by this organism indicated that CO_2 was also formed, initial efforts were made

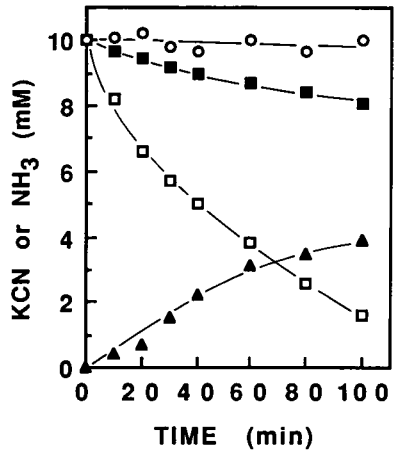

FIG. 2. Biotransformation of 10 mM KCN by washed-cell suspensions (40 mg ml^{-1} [dry weight]) of *P. fluorescens* NCIMB 11764. Symbols: □ and ▲, KCN consumption and ammonia formation, respectively, by washed cells incubated aerobically after growth on cyanide as the sole nitrogen source; ■, KCN consumed by cyanide-grown cells incubated anaerobically; ○, KCN consumed by ammonia-grown cells incubated aerobically.

to confirm these findings by ^{13}C NMR analysis. Figure 3 shows a typical ^{13}C NMR spectrum of products detected in an incubation mixture supplied with 50 mM $K^{13}CN$. Three chemical species, designated metabolites 1 to 3, giving resonance signals of 160.49 ± 0.04, 166.96 ± 0.10, and 171.10 ± 0.10 ppm, respectively, were detected. A single resonance signal corresponding to the protonated form of cyanide ($H^{13}CN$) was detected at 124 ppm. The chemical shift of cyanide varied somewhat, since the ratio of protonated to ionic species ($H^{13}CN/^{13}CN^-$) is concentration dependent. Metabolite 1 (160.49 ± 0.04 ppm) was identified as $H^{13}CO_3^-$ by comparison with an authentic standard prepared as described in Materials and Methods. Chemical shift values for metabolites 2 and 3 were shown to be identical to those obtained for authentic formamide (metabolite 2, 166.96 ± 0.10) and formate (metabolite 3, 171.10 ± 0.10). These values were further shown to be the same as those reported in the literature (19, 25, 28). In addition, both biological products showed a doublet pattern when analyzed in the gated decoupled mode, consistent with the expected coupling between a single carbon and a proton nucleus found in each compound. Coupling constants ($J_{^{13}C-^1H}$; Fig. 3) were further found to be the same as those for authentic compounds and as described previously (1, 11). Biologically produced bicarbonate (metabolite 1), as expected, showed no coupled spectra. While all three C_1 metabolites were routinely observed in incubation mixtures supplied with 50 mM KCN, at lower substrate concentrations (5 to 10 mM) bicarbonate represented the major species present.

Further identification and recovery of cyanide transformation products. In addition to ^{13}C NMR analysis, other analytical methods were used to verify the identities of formamide and formate in biological reaction mixtures. Routine quantitation of both compounds was accomplished by either colorimetric or enzymatic methods as described in Materials and Methods. Their presence in biological reaction mixtures was further confirmed by HPLC. Samples typically derived from incubation mixtures supplied with 50 mM KCN showed in HPLC two species having elution times of 33.3 and 18.3 min (data not shown). These were identified as formamide and formate, respectively, by comparison with authentic standards chromatographed under identical conditions. Neither compound was detected when washed-cell

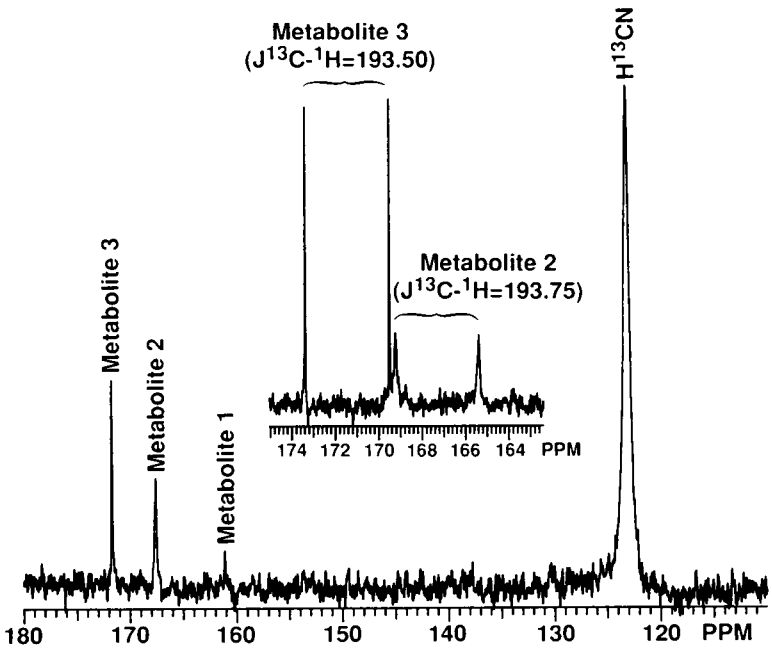

FIG. 3. ^{13}C NMR spectrum of reaction products (metabolites 1 to 3) generated from 50 mM $K^{13}CN$ (3.26 mg ml^{-1}) by a washed-cell suspension (40 mg ml^{-1} [dry weight]) of *P. fluorescens* NCIMB 11764. After 30 min of incubation, cells were removed by centrifugation and the supernatant was analyzed at 50 MHz. (Inset) Coupled spectra of metabolites 2 and 3 corresponding, respectively, to biologically produced formamide and formate (*J*, Hz).

TABLE 1. Recovery of cyanide conversion products generated under aerobic conditions by *P. fluorescens* NCIMB 11764

KCN supplied (mM)[a]	Incubation time (h)	KCN consumed (mM)	Product recovered[b]				Elemental recovery (% molar yield)[c]	
			NH_3	$HCONH_2$	HCO_2H	CO_2[d]	N	C
5.0	0.6	5.0	2.2	0.9 (20.6)	0.06 (1.4)	3.4 (78.0)	62.0	87.2
10.0	1.4	10.0	5.5	2.2 (22.6)	0.34 (3.5)	7.2 (74.0)	77.0	97.4
20.0	2.4	18.5	12.5	5.0 (30.7)	3.3 (20.2)	8.0 (49.1)	94.6	88.1
50.0	6.3	42.5	16.7	20.9 (67.0)	5.5 (17.6)	4.8 (15.4)	88.5	73.4

[a] Incubations were performed with 15-ml sealed serum vials containing 1 ml of cell suspension (40 mg ml^{-1} [dry weight]), with air in the gas phase.
[b] Expressed as mean values (millimolar) for three separate determinations made with the analytical procedures described in Materials and Methods. Numbers in parentheses represent the relative percentages of total carbon recovered for each metabolite.
[c] Percent molar yield for N is the sum of the molar recoveries of NH_3 and $HCONH_2$; percent molar yield for C is the sum of the molar recoveries of $HCONH_2$, HCO_2H, and CO_2.
[d] CO_2 determinations represent theoretical conversion estimates based on results obtained from radiolabelling experiments (see Fig. 4).

incubations were performed with boiled cells (10 min at 95°C) or with cells incubated in the absence of substrate.

Table 1 shows the results obtained when cell suspensions were supplied with different concentrations of KCN and the products quantitated when reactions were essentially complete. The time required for conversion, as might be expected, varied with the concentration of KCN supplied, but in each instance, ammonia, formamide, and formate could be detected. Calculations to determine the reaction stoichiometries revealed that 62 to 94.6% of the cyanide-derived nitrogen equivalents were recovered as ammonia and formamide. However, analogous efforts to account for the total amount of cyanide-derived carbon equivalents recovered (molar sum of formamide and formate) fell far short of 100%. It was hypothesized that this result might have been due to the fact that CO_2 represented an additional reaction product, as already had been indicated from separate ^{13}C NMR experiments (Fig. 3). To further verify this possibility and determine the relative yield of CO_2 in comparison with other products formed, we performed separate incubations with radiolabelled cyanide as a substrate and determined the amount of radioactivity recovered in volatile ($^{14}CO_2$) and nonvolatile (^{14}C-labelled formamide and ^{14}C-labelled formate) products. For this purpose, reactions were allowed to proceed nearly to completion, as ascertained from simultaneous colorimetric measurements for cyanide, and the amount of radioactivity recovered in different incubation fractions was determined (see Materials and Methods). The results of these experiments are summarized in Fig. 4. At 5 and 10 mM KCN, approximately 70% of the available radioactivity was recovered as a volatile product, presumed to be CO_2. In contrast, at 20 and 50 mM KCN, less CO_2 appeared to be made and most of the radioactivity (45 and 75%, respectively) was present in the nonvolatile fraction, presumed to contain labelled formamide and formate. On the basis of the results obtained from these types of experiments, it was possible to estimate the amount of nonradioactive CO_2 produced in separate incubations with nonradioactive KCN, where the amount of formamide and formate (plus ammonia) had already been determined chemically. When these values were incorporated into the data shown in Table 1, approximately 73 to 97.4% (depending on the initial substrate concentration) of the cyanide-derived carbon equivalents could be accounted for in products that included CO_2, formamide, and formate.

Effect of reaction conditions on cyanide conversion and product formation. An examination of reaction product yields revealed a significant difference in the amount of each metabolite formed at different substrate concentrations (Table 1). This difference was of particular interest when comparisons of the yields of C_1 metabolites were made. For example, at 10 mM KCN the relative formamide/formate/CO_2 product ratio was 23:4:74 (percent). By comparison, at 20 and 50 mM KCN the ratio changed to 31:20:49 and 67:18:15 (percent), respectively, reflecting the decreased yield of CO_2 at higher substrate concentrations. These results suggested that more than a single mechanism of cyanide conversion might be operative in NCIMB 11764. For example, we hypothesized that the formation of CO_2 might require oxygen, whereas the production of formamide and formate might not. Since we earlier had noted that washed cells catalyzed a slow but measurable disappearance of cyanide under anaerobic conditions (Fig. 2), it seemed appropriate to (i) determine whether prolonged anaerobic incubation might lead to the accumulation of any metabolites and (ii) compare the relative product ratios with those obtained for cells incubated aerobically. To accomplish this, we incubated cyanide-grown cells anaerobically with KCN until approximately 90% of the available substrate had been consumed, at which time reaction mixtures were analyzed for metabolic products. The results of these determinations (Table 2) showed that in each experiment, the time required

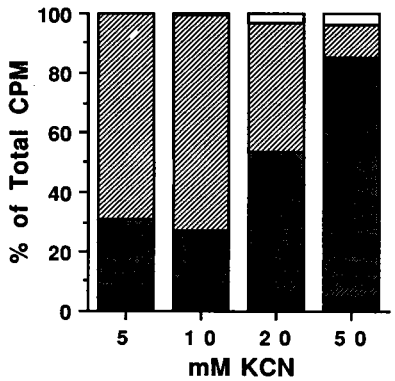

FIG. 4. Fractionation pattern for ^{14}C-labelled reaction products formed from $K^{14}CN$ by washed-cell suspensions of *P. fluorescens* NCIMB 11764. The results shown represent the mean of three separate determinations. Symbols: ▭, center-well barium soluble; ▨, center-well barium precipitate ($^{14}CO_2$); ▩, main-compartment barium soluble; ▨ main-compartment barium precipitate ($H^{14}CO_3^-$); ■, cell pellet.

TABLE 2. Recovery of cyanide conversion products generated under anaerobic conditions by *P. fluorescens* NCIMB 11764

KCN supplied (mM)[a]	Incubation time (h)	KCN consumed (mM)	Product recovered[b]				Elemental recovery (% molar yield)[c]	
			NH_3	$HCONH_2$	HCO_2H	CO_2[d]	N	C
0.5	7.0	0.5	0.26	0.22 (54.2)	0.19 (45.2)	0.01 (2.4)	96.0	84.0
10.0	12.5	8.9	4.0	5.0 (56.2)	3.8 (42.7)	0.08 (0.9)	101.1	99.8

[a] Incubations were performed in 15-ml sealed serum vials containing 1 ml of cell suspension (40 mg ml^{-1} [dry weight]), with the air atmosphere having been replaced with N_2.
[b] Expressed as mean values (millimolar) for three separate determinations. Numbers in parentheses represent the relative percentages of total carbon recovered for each metabolite.
[c] Percent molar yield for N is the sum of the molar recoveries NH_3 and $HCONH_2$; percent molar yield for C is the sum of the molar recoveries of $HCONH_2$, HCO_2H, and CO_2.
[d] CO_2 determinations represent theoretical conversion estimates based on results obtained from separate radiolabelling experiments (data not shown).

for complete conversion of KCN was considerably longer than that in comparable aerobic incubations (Table 1). In each instance, however, ammonia, formamide, and formate could again be detected. Interestingly, no significant production of CO_2 (determined from simultaneous incubations performed with radioactive $K^{14}CN$) was observed. Product balance calculations further revealed that essentially 100% of both cyanide-derived carbon and cyanide-derived nitrogen equivalents could be accounted for, respectively, as formamide and formate and as ammonia and formamide. Moreover, a comparison of product ratios with those obtained under aerobic conditions revealed a significant difference, particularly with respect to carbon metabolites. In this case, only trace amounts of CO_2 were produced, while formamide and formate were present in almost equimolar proportions. The yields of ammonia were further found to exceed those observed under aerobic conditions, presumably reflecting enhanced accumulation due to a lack of metabolism under anaerobic conditions.

Metabolism of formamide. The detection of formamide as a cyanide conversion product prompted experiments to determine whether this compound could be further metabolized. Initial experiments to address this question involved testing cells for the ability to grow on formamide as a nitrogen source. These tests consistently yielded negative results. Further attempts to show that formamide was degraded by either whole-cell suspensions or cell extracts of cyanide-grown cells also proved unsuccessful, even following prolonged incubation (10 h).

DISCUSSION

Earlier studies of cyanide metabolism by *P. fluorescens* NCIMB 11764 conducted by Harris and Knowles (8, 10) revealed that the cultivation of cells on cyanide as the sole nitrogen source could be achieved under fed-batch conditions in which the concentration of cyanide was not allowed to build up to toxic levels. A related fed-batch procedure for the cultivation of cells was also used in the present work (Fig. 1). This procedure, based on carefully timed additions of KCN to batch cultures, resulted in the rapid consumption of cyanide, but growth, as measured by increases in cell density, lagged somewhat behind. We interpret these growth kinetics to mean that several metabolic events may be involved in the assimilation of cyanide as a growth substrate. Earlier reports (8, 10) that cyanide was converted to ammonia during growth could not be duplicated, probably because, once formed, ammonia is rapidly metabolized and does not accumulate.

Although ammonia accumulation during growth was not observed, the identification of ammonia as a biological reaction product was successfully accomplished in washed-cell experiments. Under these conditions, molar yields of ammonia ranging from 40% to almost 70% were observed (Table 1). The formation of ammonia was further shown to occur in concert with cyanide consumption (Fig. 2). These findings are therefore consistent with earlier reports describing ammonia as an end product of cyanide conversion by strain NCIMB 11764. Its identification further helps to explain how cyanide can serve as a provisional nitrogen source, since ammonia is readily assimilated by this organism.

Additional experiments with washed-cell suspensions revealed that cyanide-grown cells of NCIMB 11764 were capable of catalyzing the conversion of cyanide at concentrations far exceeding those described in previous investigations. For example, KCN supplied at concentrations as high as 100 mM (6,510 ppm) was removed from incubation mixtures. Preliminary indications are that cyanide is degraded at rates approaching 2 to 5 nmol min^{-1} mg^{-1} of cell dry weight, depending on the substrate concentration. Results which showed that conversion was markedly reduced under anaerobic conditions and did not occur when cells were cultivated on ammonia (Fig. 2) are consistent with earlier observations (8, 10, 16), from which it was concluded that cyanide transformation by NCIMB 11764 is both an inducible process and an oxygen-dependent process. The identification of CO_2 as the major carbon-containing conversion product by both ^{13}C NMR analysis (Fig. 3) and radioisotopic trapping experiments (Fig. 4) also supports previous work in which this compound was identified as a major reaction product. However, this is the first report demonstrating that CO_2 and ammonia are not the only products of cyanide conversion by this organism. In addition, formamide and formate were also identified by ^{13}C NMR spectroscopy (Fig. 3), HPLC analysis, and other analytical methods. This discovery, taken together with data on product stoichiometries determined under different reaction conditions (Tables 1 and 2), provides strong evidence that additional pathways besides the putative oxygenase-mediated mechanism proposed previously (2, 9, 10, 15, 16) can be elaborated by strain NCIMB 11764. We propose three possible mechanisms of cyanide conversion by this organism (Fig. 5). The first is consistent with the idea that CO_2 and ammonia represent reaction products formed by an oxygen-dependent pathway. Evidence in support of this idea comes from the finding that CO_2 was the major C_1 metabolite detected in aerobic incubations supplied with relatively low concentrations of KCN

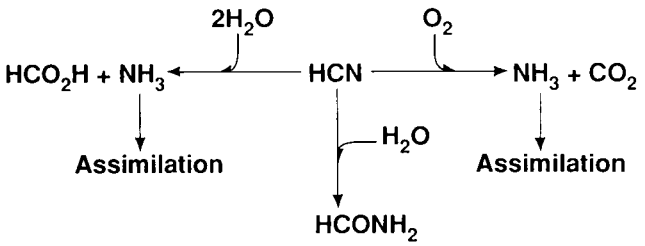

FIG. 5. Metabolic pathways of cyanide conversion by *P. fluorescens* NCIMB 11764.

(0.5 to 10 mM) (Fig. 4 and Table 1). In contrast, at higher KCN concentrations (20 and 50 mM), formamide and formate were principally formed, indicating that the route to CO_2 under these conditions is less significant. The possibility that high concentrations of KCN also inhibit the formation of CO_2 also exists.

The two alternative routes of cyanide metabolism proposed include (i) conversion to formate and ammonia and (ii) conversion to formamide. In addition to the detection of both formamide and formate in aerobic incubations, these compounds represented essentially the only carbon-derived products when cells were incubated with KCN anaerobically (Table 2). These findings therefore indicate that NCIMB 11764 can metabolize cyanide by mechanisms that are oxygen independent. The fact that both formamide and formate were always simultaneously present in incubation mixtures, as opposed to one metabolite being detected to the exclusion of the other, points toward there being two separate pathways of conversion, one for each. Furthermore, the inability to demonstrate that formamide could support growth or was metabolized by cell suspensions and cell extracts of cyanide-grown cells further suggests that it accumulates as an end product and is not further metabolized. Thus, rather than being a precursor of formate, formamide is thought to arise by a separate mechanism, as depicted in Fig. 5.

The present work demonstrates that bacteria may be able to elaborate several mechanisms of cyanide degradation. Subsistence on this compound as the sole nitrogen source appears to be relatively straightforward, involving chemical transformation to ammonia. However, the enzymatic basis of this process in *P. fluorescens* NCIMB 11764 is still only moderately well understood. Aerobic conversion of cyanide to CO_2 and ammonia could very well be mediated by an oxygenase-type enzyme, as proposed earlier, but unequivocal proof for this has yet to emerge. The pathway leading to formate is formally similar to the nitrilase (EC 3.5.5.1) catalysis pathway, involving direct cyanide hydrolysis. This conversion resembles that described for *Alcaligenes xylosoxidans* subsp. *denitrificans*, for which the enzyme description cyanidase was proposed (13); it is also similar to that reported for the cyanide-utilizing *Pseudomonas* species isolated by White et al. (28). Finally, conversion to formamide is analogous to the cyanide transformation described for phytopathogenic fungi and mediated by the enzyme cyanide hydratase (EC 4.2.1.66) (7, 15). This is the first report, to our knowledge, that bacteria are also able to carry out this transformation. The reasons why *P. fluorescens* NCIMB 11764 forms more than one cyanide-degrading system are not known, but together these systems undoubtedly provide a strong selection advantage for both detoxification and nutritional assimilation. Further insights into this question will await the resolution and further characterization of the responsible enzymes.

ACKNOWLEDGMENTS

We thank Michael Richmond for assistance with ^{13}C NMR spectroscopy and helpful discussions. The valuable suggestions and criticisms provided by P. J. Weimer are also gratefully acknowledged. L. Barber assisted with the preparation of the manuscript.

This work was supported by grant DCB 9104601 from the National Science Foundation, by Public Health Service Biomedical Research Support grant 2S07RR07195, and by the University of North Texas Organized Faculty Research Fund.

REFERENCES

1. **Dorman, D. E., and F. A. Bovey.** 1973. Proton coupled carbon-13 magnetic resonance spectra. The simple amides. J. Org. Chem. **38:**1719–1722.
2. **Dorr, P. K., and C. J. Knowles.** 1989. Cyanide oxygenase and cyanase activities of *Pseudomonas fluorescens* NCIMB 11764. FEMS Microbiol. Lett. **60:**289–294.
3. **Fallon, R. D., D. A. Cooper, R. Speece, and M. Henson.** 1991. Anaerobic biodegradation of cyanide under methanogenic conditions. Appl. Environ. Microbiol. **57:**1656–1662.
4. **Fawcett, J. K., and J. E. Scott.** 1960. A rapid and precise method for the determination of urea. J. Clin. Pathol. **13:**156–159.
5. **Finneagan, J., S. Toerien, L. Abbot, F. Smit, and H. G. Raubenheimer.** 1991. Identification and characterization of an *Acinetobacter* species capable of assimilation of a range of cyano-metal complexes, free cyanide and simple organic nitriles. Appl. Microbiol. Biotechnol. **36:**142–144.
6. **Fry, W. E., and R. L. Millar.** 1972. Cyanide degradation by an enzyme from *Stemphylium loti*. Arch. Biochem. Biophys. **151:** 468–474.
7. **Fry, W. E., and D. F. Myers.** 1981. Hydrogen cyanide metabolism by fungal pathogens of cyanogenic plants, p. 321–334. *In* B. Vennesland et al. (ed.), Cyanide in biology. Academic Press Inc., London.
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., A. W. Bunch, and C. J. Knowles.** 1987. Microbial cyanide and nitrile metabolism. Sci. Prog. **71:**293–304.
10. **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. FEMS Microbiol. Lett. **20:**337–341.
11. **Hinton, J. F., and K. H. Ladner.** 1972. NMR studies of the structure of aqueous amide solutions. J. Magn. Reson. **6:**586–599.
12. **Höpner, T., and J. Knappe.** 1974. Formate: determination with formate dehydrogenase, p. 1551–1555. Methods of enzymatic analysis, vol. 3. Academic Press Inc., New York.
13. **Ingvorsen, K., B. Højer-Pedersen, and S. E. Godtfredsen.** 1991. Novel cyanide-hydrolyzing enzyme from *Alcaligenes xylosoxidans* subsp. *denitrificans*. Appl. Environ. Microbiol. **57:**1783–1789.
14. **Knowles, C. J.** 1976. Microorganisms and cyanide. Bacteriol. Rev. **40:**652–680.
15. **Knowles, C. J.** 1988. Cyanide utilization and degradation by microorganisms. CIBA Found. Symp. **140:**3–15.
16. **Knowles, C. J., and A. W. Bunch.** 1986. Microbial cyanide metabolism. Adv. Microb. Physiol. **27:**73–111.
17. **Kunz, D. A., and O. Nagappan.** 1989. Cyanase-mediated utilization of cyanate in *Pseudomonas fluorescens* NCIMB 11764. Appl. Environ. Microbiol. **55:**256–258.
18. **Lambert, J. L., J. Ramasamy, and J. V. Paukstelis.** 1975. Stable reagents for the colorimetric determination of cyanide by modified König reactions. Anal. Chem. **47:**916–918.
19. **Levy, G. C., and G. L. Nelson.** 1972. Carbon-13 nuclear magnetic resonance for organic chemists, p. 109–135. John Wiley & Sons, Inc., New York.
20. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
21. **Neidhardt, F. C., J. L. Ingraham, and M. Schaechter.** 1990. Physiology of the bacterial cell: a molecular approach. Sinauer

Associates Inc., Publishers, Sunderland, Mass.
22. **Rollinson, G., R. Jones, M. P. Meadows, R. E. Harris, and C. J. Knowles.** 1987. The growth of a cyanide-utilizing strain of *Pseudomonas fluorescens* in liquid culture on nickel cyanide as a source of nitrogen. FEMS Microbiol. Lett. **40:**199–205.
23. **Silva-Avalos, J., M. G. Richmond, O. Nagappan, and D. A. Kunz.** 1990. Degradation of the metal-cyano complex tetracyanonickelate(II) by cyanide-utilizing bacterial isolates. Appl. Environ. Microbiol. **56:**3664–3670.
24. **Snell, F. D., and C. T. Snell.** 1954. Aliphatic amines and amides, p. 29–33. *In* Colorimetric methods of analysis, 3rd ed., vol. IV. D. Van Nostrand Co. Inc., New York.
25. **Stothers, J. B.** 1972. Carbon-13 NMR spectroscopy, p. 295–308. *In* A. T. Blomquist and H. Wasserman (ed.), Organic chemistry, vol. 24. Academic Press Inc., New York.
26. **Tyler, B.** 1978. Regulation of the assimilation of nitrogen compounds. Annu. Rev. Biochem. **47:**1127–1162.
27. **Ware, G. C., and H. A. Painter.** 1955. Bacterial utilization of cyanide. Nature (London) **175:**900.
28. **White, J. M., D. D. Jones, D. Huang, and J. J. Gauthier.** 1988. Conversion of cyanide to formate and ammonia by a pseudomonad obtained from industrial wastewater. J. Ind. Microbiol. **3:**263–272.
29. **Winter, J. A.** 1963. The use of a specific actinomycete to degrade cyanide wastes, p. 703–716. *In* Proceedings of the 18th Industrial Waste Conference. Purdue University, West Lafayette, Ind.