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Novel Cyanide-Hydrolyzing Enzyme from *Alcaligenes xylosoxidans* subsp. *denitrificans*

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A cyanide-metabolizing bacterium, strain DF3, isolated from soil was identified as *Alcaligenes xylosoxidans* subsp. *denitrificans*. Whole cells and cell extracts of strain DF3 catalyzed hydrolysis of cyanide to formate and ammonia ($HCN + 2H_2O \rightarrow HCOOH + NH_3$) without forming formamide as a free intermediate. The cyanide-hydrolyzing activity was inducibly produced in cells during growth in cyanide-containing media. Cyanate (OCN^-) and a wide range of aliphatic and aromatic nitriles were not hydrolyzed by intact cells of *A. xylosoxidans* subsp. *denitrificans* DF3. Strain DF3 hydrolyzed cyanide with great efficacy. Thus, by using resting induced cells at a concentration of 11.3 mg (dry weight) per ml, the cyanide concentration could be reduced from 0.97 M (approximately 25,220 ppm) to less than 77 nM (approximately 0.002 ppm) in 55 h. Enzyme purification established that cyanide hydrolysis by *A. xylosoxidans* subsp. *denitrificans* DF3 was due to a single intracellular enzyme. The soluble enzyme was purified approximately 160-fold, and the first 25 NH_2-terminal amino acids were determined by automated Edman degradation. The molecular mass of the active enzyme (purity, >97% as determined by amino acid sequencing) was estimated to be >300,000 Da. The cyanide-hydrolyzing enzyme of *A. xylosoxidans* subsp. *denitrificans* DF3 was tentatively named cyanidase to distinguish it from known nitrilases (EC 3.5.5.1) which act on organic nitriles.

While cyanide occurs naturally at low levels in plants and microorganisms (6, 7, 14, 16, 21), concentrated amounts are due to human activities. Industries dealing with metal plating and finishing, production of synthetic fibers, and mining and extraction of metals generate wastes containing large quantities of cyanide (14, 23, 32). Wastes produced by industries processing cyanogenic crops (e.g., cassava and bitter almonds) also contain considerable amounts of cyanide originating from the decomposition of cyanogenic glucosides in the plant material.

Cyanide is a potent inhibitor of cellular metabolism (3, 14, 16, 28), and cyanide in industrial process wastewater must be reduced to low levels, generally ≤4 to 40 μM (~0.1 to 1.0 ppm), before the wastewater can be discharged (32). Many chemical processes currently used to detoxify cyanide-containing industrial wastes suffer from drawbacks (14, 31). For instance, alkaline chlorination, a widely used process, requires careful control of the chlorine concentration and may give rise to uncontrolled formation of toxic and biologically persistent organochlorine compounds, thus producing effluents requiring additional treatment. Accordingly, research efforts have been devoted towards development of chlorine-free detoxification techniques (23, 32), with one area of research being microbial treatment of cyanide effluents (15).

Microorganisms are known to possess various enzymes capable of converting cyanide into compounds which may serve as carbon and nitrogen substrates. Such enzymes include formamide hydro-lyase (EC 4.2.1.66), L-3-cyanoalanine synthase (EC 4.4.1.9), thiosulfate sulfurtransferase (EC 2.8.1.1), and oxygenases (8, 26; see references 14 and 16 for recent reviews). Recently, a report by White et al. describing microbial hydrolysis of cyanide directly to formate and ammonia by a *Pseudomonas* sp. was published (31). Although the enzyme involved was not purified, their data provide strong evidence that cyanide hydrolysis was catalyzed by a single enzyme.

Various types of acclimated microbial systems (e.g., activated sludge systems and trickling filters) for treatment of cyanide-containing wastewaters have been reported (14, 16, 25, 31, and 32 and references cited therein). In most of these cases, the microorganisms responsible for cyanide detoxification were not identified or studied in detail.

Biological systems may require long adaptation and apparently do not function properly when exposed to cyanide concentrations higher than about 8 mM (14, 25, 31). Enzymatic removal of cyanide may therefore offer some advantages over currently applied biological treatment processes, as the enzymes can work directly in raw or concentrated wastewaters containing cyanide levels too toxic for microbial growth (17, 28).

Published data indicate that some cyanide-converting enzymes are subject to cyanide inhibition (9, 22, 27, 31). Information regarding their performance at high cyanide concentrations or their ability to remove trace levels of cyanide is limited.

The present work was undertaken with the dual purpose of isolating new types of cyanide-metabolizing microorganisms and investigating enzymatic cyanide degradation under extreme cyanide concentrations.

(Some of these results were presented previously [10a].)

MATERIALS AND METHODS

Enrichment and culture conditions. Cyanide-metabolizing microorganisms were isolated by enrichment culture from soil and water samples in a liquid isolation medium containing sodium cyanide (5 mM) as the nitrogen source. The enrichment medium (BS medium) had the following ingredients (amounts given per liter): $Na_2HPO_4 \cdot 2H_2O$, 7 g; KH_2PO_4, 3 g; NaCl, 0.25 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; $FeCl_3 \cdot 6H_2O$, 0.045 g; $MnSO_4 \cdot 4H_2O$, 0.01 g; $ZnSO_4 \cdot 7H_2O$, 0.01 g; $CuSO_4 \cdot 5H_2O$, 0.002

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g; $CoCl_2 \cdot 6H_2O$, 0.003 g; $NiCl_2 \cdot 6H_2O$, 0.003 g; and $Na_2MoO_4 \cdot 2H_2O$, 0.002 g. The pH of the medium was adjusted to 7.5 before sterilization by using sodium hydroxide or phosphoric acid. Acetate, glycerol, glucose, sucrose, or combinations thereof were used as the carbon and energy sources at final concentrations of 0.25% (wt/vol). The carbon and energy substrates were autoclaved directly in the isolation medium, with the exception of carbohydrates, which were autoclaved separately. Sodium cyanide was added from a filter-sterilized (0.2-μm-pore-size filter) solution to the cooled medium immediately before inoculation. Cotton-plugged shake flasks with BS medium were inoculated with environmental samples and incubated aerobically at 25°C on a rotary shaker (230 rpm) placed in a hood. During the enrichment procedure, fresh cyanide solution was added to the shake flasks at 48-h intervals to compensate for cyanide loss due to air stripping. Enrichment cultures showing growth and depletion of cyanide were subcultured three times in fresh BS medium. Individual cyanide-transforming microorganisms were isolated by streaking on solid (1.5% agar) BS medium supplemented with 0.05% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.) and incubated in a desiccator at 25°C. Purity was confirmed by plating on nutrient broth medium (Difco). The ability of purified isolates to metabolize cyanide was verified with washed cell suspensions as described below.

Strain DF3, described below, was maintained at 4°C on slopes of nutrient broth (Difco). Large quantities of cells were obtained by aerobic culture at 30°C in nutrient broth supplemented with 1.0% (wt/vol) glycerol and 5 mM sodium cyanide (NBG medium). Growth was monitored by measuring the optical density at 640 nm. Enzyme yields in NBG medium were typically 0.13 to 0.25 U per mg (dry weight) of cells after 30 to 40 h of cultivation. The growth yield in NBG medium was approximately 3.4 mg (dry weight) of cells per ml of medium, which corresponds to 8.7 units of optical density at 640 nm.

Harvesting and preparation of cell extracts. Cells grown for 30 to 40 h were harvested by centrifugation (14,000 × *g* for 15 min at 4°C). The pellet was washed twice in 0.1 M Na_2HPO_4-NaH_2PO_4 buffer (pH 7.8) and resuspended in a small volume of the same buffer. This suspension of resting cells was used to assay cyanide-degrading activity. Washed cells of strain DF3 could be stored at 4°C for 2 weeks without loss of cyanide-degrading activity. Cell extracts of strain DF3 were prepared by disrupting washed cells in a model LAB 12.51H high-pressure homogenizer (Rannie A/S, Albertslund, Denmark) fitted with a cooling device. Cell disruption was carried out at 95,000 kPa. Following disruption, the mixture was centrifuged at 23,000 × *g* for 30 min at 4°C. The supernatant fluid (cell extract) was stored at −25°C until required (less than 7 days).

Assay of CHA. Determination of cyanide-hydrolyzing activity (CHA) was based on detection of the disappearance of cyanide in reaction mixtures containing washed whole cells or cell extracts. The reaction mixture (final pH 7.8) for determination of CHA contained 50 mM sodium cyanide in 0.1 M Na_2HPO_4–NaH_2PO_4 buffer and an appropriate amount of enzyme sample at a concentration of 0.2 to 0.6 U/ml. The enzymatic reaction was started by addition of the substrate and carried out at 25°C in closed test tubes mounted on a rotator. Samples were quickly removed from the reaction mixture at appropriate intervals (i.e., before 10% of the substrate had been converted), and the reaction was stopped by addition of a small volume of 6 M NaOH (final pH > 11). The reaction mixture was then centrifuged, and the supernatant was filtered (0.2-μm-pore-size filters) before chemical analysis.

Whole cells and purified enzyme preparations from *Alcaligenes xylosoxidans* subsp. *denitrificans* DF3 produced ammonia in nearly stoichiometric amounts from cyanide. The CHA of resting cells of DF3 cells was, therefore, routinely determined by measuring the appearance of ammonia. Control reactions in mixtures containing washed cells and phosphate buffer were run for each incubation and used to correct for ammonia release from cells. No significant hydrolysis of cyanide was detected in reaction mixtures devoid of enzyme. One unit of CHA is defined as the amount of enzyme (whole cells or purified enzyme) which catalyzes the formation of 1 μmol of ammonia in 1 min under the conditions described above.

Analytical methods. The amounts of cyanide in reaction mixtures and culture broth were assayed colorimetrically by method 412D of the *Standard Methods for the Examination of Water and Wastewater* (1) or with a Spectroquant 14797 test kit (E. Merck, Darmstadt, Germany) by a Shimadzu Graphicord UV 240 spectrophotometer. Unless stated otherwise, the term cyanide refers to CN^- + HCN. Ammonia was measured colorimetrically by the indophenol method as described by Chaney and Marbach (4). Cyanide concentrations of <50 mM were found not to interfere with the indophenol reaction when 10- or 50-μl sample volumes and 1 ml each of reagents 1 and 2 were used. Cyanide concentrations of ≥100 mM seriously impaired color formation. Formate and formamide were assayed by analytical high-pressure liquid chromatography (HPLC) using a Shimadzu LC-6A system equipped with an HPX-87H ion-exchange column (300 by 7.8 mm; Bio-Rad Laboratories, Richmond, Calif.). The mobile phase consisted of double-distilled water adjusted to pH 2.0 with dilute H_2SO_4. Elution was isocratic at a flow rate of 0.5 ml/min at 22°C. The A_{210} was measured with a Shimadzu SPD-6A UV spectrophotometric detector coupled to a Shimadzu C-R3A integrator. All samples were filtered (0.2-μm-pore-size filter) to remove cells and other particulates before analysis. The amount of protein in cell extracts was estimated by the method of Lowry et al. (19) with bovine serum albumin (Sigma, St. Louis, Mo.) as the standard.

Purification of CHA from strain DF3. Washed cells of *A. xylosoxidans* subsp. *denitrificans* DF3 were suspended in 0.1 M potassium phosphate buffer (pH 7.0) at 2°C and disrupted at 95,000 kPa in the Rannie homogenizer. Intermittent cooling with dry ice was applied to ensure that the temperature did not rise above 25°C. After homogenization, the cell debris was removed by centrifugation (23,000 × *g* for 30 min at 4°C), and the resultant cell extract was treated with a 50 mg/ml protamine sulfate solution in water (10%, vol/vol). The resulting mixture was centrifuged (4,000 × *g* for 15 min at 4°C), and the CHA was subsequently redissolved in 0.25 volume of 0.1 M phosphate buffer and applied onto a DEAE–Sepharose CL-6B column (Pharmacia/LKB, Uppsala, Sweden) previously equilibrated with 0.1 M phosphate buffer (pH 7.0). Proteins bound to the column were eluted with a sodium chloride gradient from 0 to 0.7 M in the phosphate buffer. All active fractions were pooled, and sodium chloride was removed by diafiltration with 0.1 M phosphate buffer (pH 7.0) on an Amicon Hollow Fiber System (DC2) at 2°C. The desalted enzyme solution was again applied onto a DEAE–Sepharose CL-6B column, and the activity was eluted with a sodium chloride gradient (0 to 0.7 M) as described above. The top fractions from the column (specific activities between 53 and 58 U/mg of

protein) were concentrated eight times by ultrafiltration in an Amicon cell equipped with a GR61PP membrane (DDS, Nakskov, Denmark; approximate molecular weight cutoff of 20,000). The resulting concentrate was applied onto a Sephacryl S-300 gel filtration column (Pharmacia/LKB) equilibrated with 0.1 M phosphate buffer (pH 7.0) by using a loading volume of approximately 2% of the bead volume (flow rate, 16 cm/h). The top fractions eluting in the void volume from the Sephacryl S-300 column were combined and are referred to as purified cyanidase. Samples of purified cyanidase were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing, gel filtration, and NH_2-terminal sequencing.

SDS-PAGE. SDS-PAGE was carried out in a 12 to 16% polyacrylamide linear gradient gel as described by Poduslo and Rodbard (24). Protein was detected by staining the gel with 0.2% Coomassie brilliant blue in 50% ethanol–10% acetic acid.

Isoelectric focusing. Isoelectric focusing was carried out on Ampholine PAG plates (Pharmacia/LKB) with a pH range of 4.0 to 6.5.

NH_2-terminal sequencing. The NH_2-terminal amino acid sequence of purified cyanidase was determined by automated Edman degradation according to the method of Thim et al. (29).

Chemicals. Chemicals and reagents used were purchased from Merck, Sigma, or Pharmacia/LKB and were of the highest commercially available grade. Yeast extract and nutrient broth were obtained from Difco. Molecular weight standards were from Pharmacia/LKB.

RESULTS

Characteristics of *A. xylosoxidans* subsp. *denitrificans* DF3. Several bacterial strains which exhibit cyanide-transforming activity were isolated by enrichment on the cyanide-containing BS medium. One bacterial strain, designated DF3, was chosen for further studies on the basis of its high level of cyanide-transforming activity. Bacteria of this strain were small, gram-negative, nonsporeforming, motile, peritrichous rods (0.5 to 0.7 μm by 0.9 to 2.5 μm). This strain reacted positively in the following tests: nitrate reduction to nitrite, nitrate reduction to N_2, oxidase, catalase, aminopeptidase (Cerny), and tyrosine degradation. The following tests were negative: urease; lecithinase; phenylalanine deaminase; levan from sucrose; Voges-Proskauer; indole fermentation; oxidation-fermentation (glucose); acid from glucose, fructose, and xylose; and hydrolysis of casein, starch, gelatin, agar, DNA, esculin, and Tween 80. The strain is able to grow with acetate, adipate, citrate, malate, phenylacetate, L-serine, or glycerol as the sole carbon and energy source. Fructose, glucose, mannose, maltose, xylose, gluconate, and mannitol do not support growth. The strain grows at 37 but not 41°C. Strain DF3 was identified by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) as a strain of *A. xylosoxidans* subsp. *denitrificans*, formerly *A. denitrificans* subsp. *denitrificans* (13). The DNA base composition (G+C content) was not determined in connection with identification of the strain.

Cyanide metabolism of strain DF3. Cyanide metabolism of *A. xylosoxidans* subsp. *denitrificans* DF3 was initially studied with harvested cyanide-grown cells. Upon addition of sodium cyanide (60 mM, dissolved in 0.1 M sodium phosphate buffer, pH 7.5) to washed cell suspensions of exponential- or stationary-phase cultures, formate and ammonia were produced stoichiometrically in a ratio of 1:1. Complete conversion of cyanide to formate and ammonia occurred under both aerobic and strictly anaerobic (N_2 atmosphere) conditions. Formamide could not be detected by HPLC (detection limit, 0.1 mM) during hydrolysis of cyanide by resting cells or cell extracts of strain DF3, thus indicating direct hydrolysis of cyanide to formate (i.e., $HCN + 2H_2O \rightarrow HCOOH + NH_3$) without formamide being a free intermediate.

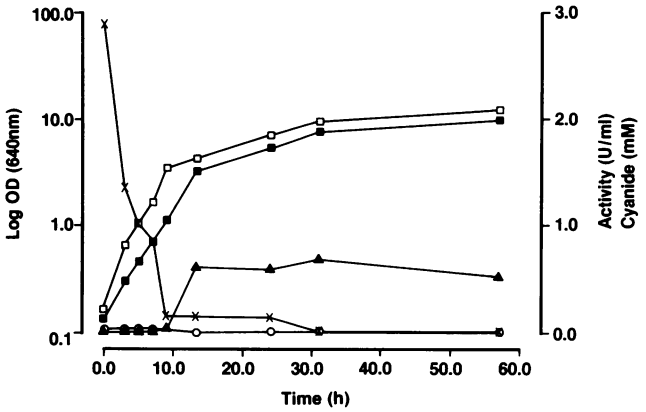

FIG. 1. Growth and enzyme induction of *A. xylosoxidans* subsp. *denitrificans* DF3 in NBG medium in the presence and the absence of cyanide. Growth conditions: 100 ml of medium in 500-ml Erlenmeyer flasks on a rotary shaker (220 rpm) at 30°C. The initial concentration of sodium cyanide was 2.9 mM. A 2% (vol/vol) inoculum of a culture grown to stationary phase (50 to 60 h after inoculation) for several passages in cyanide-free NBG medium was used to inoculate both experimental flasks. Symbols for induced culture: ■, optical density (OD); ×, cyanide concentration in growth medium; ▲, CHA. Symbols for uninduced culture: □, OD; ○, CHA.

The cyanide-metabolizing activity of strain DF3 is induced by sodium cyanide during growth in BS medium and various complex media (e.g., nutrient broth). Enzyme biosynthesis is not repressed during growth in media containing high concentrations of ammonia, organic nitrogen compounds, or both. A typical time course of the cultivation of strain DF3 is shown in Fig. 1. Strain DF3 grew exponentially during the first 10 h in the presence of 3 to 0.3 mM NaCN, and the CHA was maximal (approximately 0.24 U/mg [dry weight] of cells at the onset of the stationary growth phase. Since cyanide is volatile at pH 7.5 and 30°C, cyanide evaporated from the growth medium during incubation on the rotary shaker. Thus, in a control experiment, the following cyanide concentrations were measured in sterile medium incubated under the same conditions as those described in the legend to Fig. 1: 2.89 mM, 0 h; 2.12 mM, 3.0 h; 1.92 mM, 5.0 h; 1.54 mM, 7.0 h; 0.65 mM, 9.0 h; 0.58 mM, 13.3 h; 0.15 mM, 24.0 h; 0.01 mM, 31.0 h; and 0.00 mM, 57 h.

Cells subcultured in NBG medium for many generations in the absence of cyanide exhibited low, but nevertheless measurable, activities in the range of 0.002 to 0.003 U/mg (dry weight) of cells (Fig. 1). Uninduced cultures were always grown in a separate incubator in order to ensure the complete absence of HCN vapors above the cultures. The presence of cyanide in the growth medium thus increased enzyme synthesis about 100-fold. It was found that cyanide concentrations in the range of 0.1 to 5 mM were equally effective for induction. Addition of a second dose of cyanide

during growth (e.g., at 30 h) did not significantly enhance enzyme expression provided that the shake flasks were inoculated with an exponentially growing culture (2%, vol/vol; results not shown). Enzyme activity was not induced when strain DF3 was grown under nitrogen limitation in batch culture. Formate, the product of cyanide hydrolysis, did not serve as an inducer of CHA.

Effects of pH and temperature. The effect of pH on the hydrolysis of cyanide by resting cells was investigated. Intact cells exhibited maximum activity at pH 7.5 to 8.2 and 35% of maximum activity at pH 9. The optimum temperature for cyanide hydrolysis by resting cells is 35 to 40°C, with a sharp decrease above 40°C.

Substrate specificity of strain DF3 cells. Cyanide-grown cell suspensions of strain DF3 did not hydrolyze cyanate (OCN^-) or any of the following organic nitriles: acetonitrile, propionitrile, acrylonitrile, methacrylonitrile, succinonitrile, adiponitrile, cyanoacetic acid, methylcyanoacetic acid, ethylcyanoacetic acid, benzonitrile, and benzyl cyanide (at concentrations of 30 mM in 0.1 M phosphate buffer [pH 7.0] at 22°C). Small amounts of ammonia were formed during incubation with mandelonitrile and lactonitrile, presumably because of enzymatic hydrolysis of free cyanide formed by spontaneous cleavage of the hydroxynitriles. Since most of the nitriles listed above and tested as substrates are known to penetrate intact cells of both gram-negative (2) and gram-positive (5, 11, 12, 20) bacteria, they are unlikely to be substrates of the cyanide-hydrolyzing enzyme system of *A. xylosoxidans* subsp. *denitrificans* DF3.

Inhibition of cyanide hydrolysis by strain DF3 cells. Preliminary results indicated that resting cells of *A. xylosoxidans* subsp. *denitrificans* DF3 were able to degrade high cyanide concentrations. Thus, experiments were conducted to determine the activity over a wide range of cyanide concentrations. A plot of the initial velocity of DF3 cell-catalyzed cyanide hydrolysis versus cyanide concentration is shown in Fig. 2A. It is apparent that enzyme activity is inhibited at cyanide concentrations above 100 mM. However, cyanide hydrolysis by intact cells of DF3 was found to exhibit zero-order kinetics for a considerable time even at high cyanide concentrations (Fig. 3). The results summarized in Fig. 3 show that DF3 cells performed complete hydrolysis of 0.97 M sodium cyanide (26,190 mg of HCN per liter) within 50 h when applied at a concentration of 2.7 U/ml (11.3 mg [dry weight] of cells per ml). The pH of the reaction mixture was 7.5 ± 0.5 throughout the experiment, during which the cells were exposed to cyanide concentrations higher than 0.5 M for approximately 22 h. The residual cyanide concentration in the reaction mixture (at 55 h) was less than 77 nM (~0.002 mg of HCN per liter). The observed hydrolysis of cyanide follows zero-order kinetics between 0 and 45 h and proceeds at a rate of 19.5 mM/h, which is approximately eight times lower than the rate observed at low cyanide concentrations when a similar dose of enzyme is used. The reduction of the hydrolysis rate is due to substrate inhibition (Fig. 2A) and, at high cyanide concentrations, to irreversible inactivation of the enzyme. Thus, when assayed after completion of the experiment whose results are illustrated in Fig. 3, the cells exhibited only 38% of their initial activity.

The effect of formate concentration on cyanide hydrolysis by intact cells was investigated. Cyanide hydrolysis by DF3 cells was found to be subject to inhibition by sodium formate (Fig. 2B), but product inhibition by formate was much less severe than cyanide inhibition (Fig. 2A). The rate of cyanide hydrolysis by DF3 cells was not affected by ammonium sulfate concentrations below 5 M, and the cyanide concentration could be reduced to less than 230 nM (~0.006 ppm) in a 5 M ammonium sulfate solution. Whole cells and crude cell extracts of strain DF3 did not function in the reverse direction (i.e., did not produce cyanide) when exposed to 3 M ammonium formate in 0.1 M phosphate buffer (pH 7.8, 22°C).

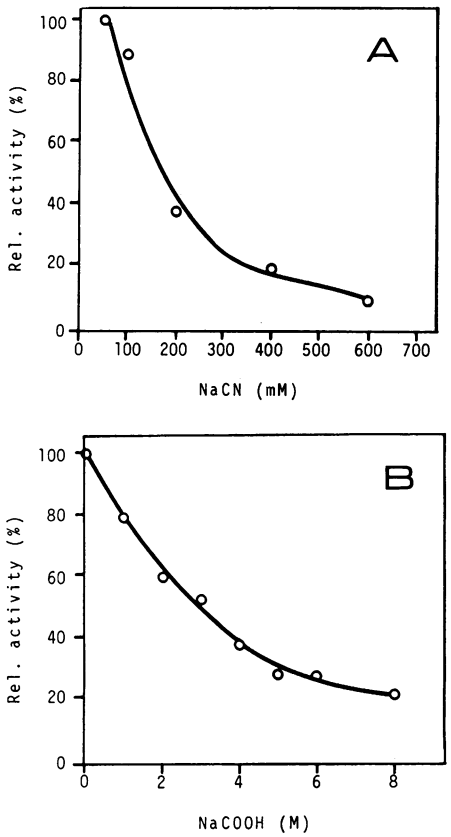

FIG. 2. Inhibition of cyanide hydrolysis by intact cells of *A. xylosoxidans* subsp. *denitrificans* DF3. (A) Inhibition by sodium cyanide. Shown in a plot of initial velocity of cyanide hydrolysis versus cyanide concentration. Initial velocities were determined at 22°C in 0.1 M sodium phosphate buffer (pH 7.5) by measuring formation of formate. Initial velocity at 50 mM NaCN = 100%. (B) Inhibition by sodium formate. Initial velocities were measured by using intact cells (1.7 mg [dry mass] of cells per ml) at 22°C. All reaction mixtures (pH adjusted to 7.5) contained 50 mM NaCN. Hydrolysis rates are expressed relative to a control without added NaCOOH. In the experiment with 8 M NaCOOH, the residual cyanide concentration was less than 230 nM at 220 h.

Cyanide hydrolysis at low cyanide concentrations. Whole-cell preparations of strain DF3 exhibit a high affinity toward cyanide, which can be depleted to very low levels. A typical time course of cyanide hydrolysis by resting cells of strain DF3 at a low cyanide concentration is shown in Fig. 4. In the experiment whose results are summarized in Fig. 4, the reaction mixture was incubated in a glass vessel with a minimum of headspace in order to minimize loss of cyanide from the solution. As in the experiment whose results are summarized in Fig. 3, the cyanide level was reduced to <77 nM after 35 h. Control experiments (without cells) carried out under identical conditions confirmed that the disappearance of cyanide from the reaction mixture is due to the enzyme-catalyzed reaction and not to chemical hydrolysis or air stripping.

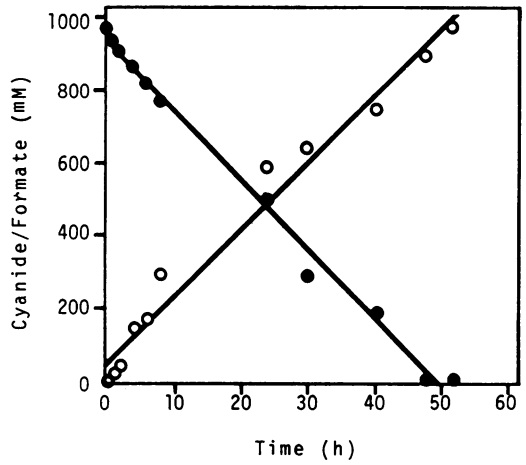

FIG. 3. Time course of enzymatic hydration of cyanide to formate with resting cells of *A. xylosoxidans* subsp. *denitrificans* DF3. The reaction mixture (50 ml) contained 970 mM NaCN in 0.1 M sodium phosphate buffer (pH 7.8) and 2.7 U of cyanidase activity per ml (11.3 mg [dry mass] of cells per ml). Incubation was at 22°C on a rotator. The loss of cyanide in a sterile control was less than 6% under identical experimental conditions. Symbols: ●, cyanide; ○, formate.

Cyanide hydrolysis by whole cells of *A. xylosoxidans* subsp. *denitrificans* did not fit simple Michaelis-Menten saturation kinetics when assayed over a broad concentration interval. This lack of conformance is due to inhibition and inactivation of the enzyme at high cyanide concentrations. However, even at low cyanide concentrations (<20 mM), at which substrate inhibition is negligible, estimates of the half-saturation constant (K_m) from progress curves varied between 1.5 and 10 mM total cyanide at pH 7.8. Thus, it is not yet possible to precisely determine the half-saturation constants for cyanide hydrolysis for cells of strain DF3. Also, it has not been established whether cyanide (CN^-) or hydrogen cyanide (HCN) serves as the true substrate.

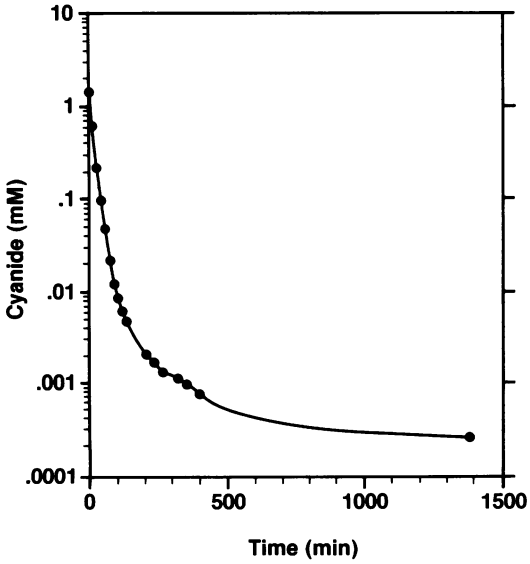

FIG. 4. Hydrolysis of cyanide (low concentration range) by intact cells of *A. xylosoxidans* subsp. *denitrificans* DF3. The reaction mixture (50 ml) contained 0.1 M sodium phosphate buffer (pH 7.8), 1.1 mM NaCN, and 0.34 U of cyanidase activity per ml (1.4 mg [dry mass] of cells per ml). Incubation was at 22°C in a closed glass vessel with low-speed magnetic stirring.

TABLE 1. Purification of cyanidase from *A. xylosoxidans* subsp. *denitrificans* DF3

Procedure	Sp act[a]	Purification (fold)
Cell extract	0.5	1.0
Protamine sulfate precipitate[b]	22	44
First DEAE–Sepharose CL-6B[c]	45	90
Second DEAE–Sepharose CL-6B[c]	58	116
Sephacryl S-300	81	162

[a] Specific activity is expressed as micromoles of ammonia liberated per milligram of protein per minute at 25°C.
[b] Redissolved in 25% of the original volume.
[c] Top fractions.

Enzyme purification. Resting cells and crude cell extracts of *A. xylosoxidans* subsp. *denitrificans* convert cyanide stoichiometrically to formate and ammonia apparently without formation of formamide as an intermediate (the detection limit for formamide by HPLC analysis was 0.1 mM). This indicates that hydrolysis of cyanide to formate proceeded by a one-step reaction, $HCN + 2H_2O \rightarrow HCOOH + NH_3$ (i.e., a mechanism similar to that of a nitrilase [EC 3.5.5.1]).

However, resting cells and crude cell extracts hydrolyze formamide to formate at about 30% the rate of cyanide, presumably by function of an amidase (EC 3.5.1.4). Accordingly, without enzyme purification, a two-step conversion of cyanide to formate and ammonia catalyzed by the sequential action of formamide hydro-lyase (EC 4.2.1.66) and formamide amidohydrolase (EC 3.5.1.49) could not be definitely ruled out. The cyanide-hydrolyzing enzyme of *A. xylosoxidans* subsp. *denitrificans* DF3 was purified about 160-fold from cell extracts (approximately 0.5 U/mg of protein), as summarized in Table 1. The overall yield was 8 to 10%. Protamine sulfate precipitation provided preparations with an activity of 22 U/mg of protein. Top fractions from the second DEAE–Sepharose CL-6B column chromatography run exhibited activities of up to 58 U/mg of protein and were applied onto a Sephacryl S-300 column. The top fractions eluting in the void volume from the Sephacryl S-300 column exhibited a specific activity of 79 to 81 U/mg of protein (171 U/ml) and are referred to as purified cyanidase. The purified cyanidase showed only two bands of equal intensity at 39,000 and 40,000 Da when examined by SDS-PAGE (Fig. 5). By isoelectric focusing, two bands with pIs of 4.55 and 4.65 could be detected. Gel filtration indicated that the active cyanidase has a molecular mass of >300,000 Da, since the enzyme eluted in the void volume.

The optimum temperature of the purified cyanidase is about 26°C (i.e., considerably lower than the 40°C activity maximum of whole cells). The optimum pH is 7.8 ± 0.2.

When NH_2-terminal sequencing of purified cyanidase was carried out, the first 25 amino acids were identified without difficulty. The NH_2-terminal sequence was found to be Met Lys Leu Arg Tyr Asn Pro Lys Phe Lys Ala Ala Ala Val Gln Ala Ser Pro Val Tyr Leu Asp Leu Gly Ala, which shows no significant homology with any enzymes of the Dayhoff protein sequence data base (National Biomedical Research Foundation, Washington, D.C.; version 19, February 1989 update), which was searched with the programs SEARCH, RELATE, and FASTP (18). The Dayhoff data base contains

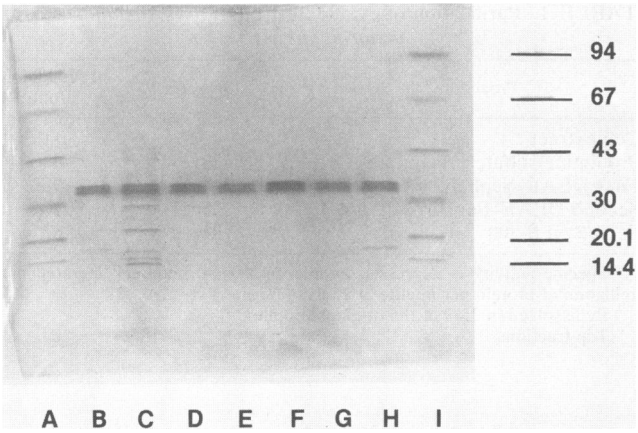

FIG. 5. SDS-PAGE of purified fractions from *A. xylosoxidans* subsp. *denitrificans* DF3 after Sephacryl S-300 column chromatography. Lanes A and I contain the following molecular weight standards: phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), α-lactalbumin (14,400). Lane B, sample applied to column (50 U/mg); lane C, fraction 17 (33 U/mg); lane D, fraction 18 (65 U/mg); lane E, fraction 19 (81 U/mg); lane F, fraction 20 (80 U/mg); lane G, fraction 21 (71 U/mg); lane H, fraction 22 (59 U/mg). The specific activities of individual fractions are given in parentheses. Specific activity is expressed as micromoles of ammonia formed per milligram of protein per minute at 25°C.

data for cyanase (cyanate hydrolase [EC 3.5.5.3]) but not for formamide hydro-lyase (EC 4.2.1.66) or nitrilase (EC 3.5.5.1). The purified cyanidase was found to be >97% pure by the sequence analysis. Since two bands of almost equal intensity at 39,000 and 40,000 Da also appeared in SDS-PAGE when other purification procedures were applied, the NH_2-terminal sequence data indicate that cyanidase may be composed of two different subunits having identical NH_2 termini. However, on the basis of the present evidence, the possibility that proteolytic degradation or, less likely, incomplete denaturation is responsible for the appearance of the 39-kDa band cannot be excluded.

Purified cyanidase converted cyanide stoichiometrically to ammonia and formate without intermediate formation of free formamide. The enzyme also did not show any activity toward formamide.

DISCUSSION

This article reports on a cyanide-degrading strain of *A. xylosoxidans* subsp. *denitrificans*, DF3, which was isolated from a soil sample. Strain DF3 was selected from a number of cyanide-metabolizing bacteria and fungi isolated in BS medium on the basis of its high level of cyanide-degrading activity and its tolerance for high cyanide concentrations. To our knowledge, this is the first report to describe the occurrence of a cyanide-converting enzyme in a member of the genus *Alcaligenes*. The type strains of *A. xylosoxidans* subsp. *denitrificans* (ATCC 15173 = NCIB 11961) did not possess constitutive or inducible cyanide-transforming activity when tested in our laboratory, nor did a number of *Alcaligenes* spp. obtained from different culture collections. The presence of CHA is, therefore, not a general characteristic of members of the genus *Alcaligenes*. Further physiological characterization and determination of the DNA base composition of strain DF3 are necessary to compare strain DF3 with previously described *Alcaligenes* species in more detail.

Resting cells and cell extracts of strain DF3 convert cyanide stoichiometrically into formate and ammonia without transient accumulation of formamide. Even though DF3 generates a formamide amidase, the purified cyanidase (specific activity, 81 U/mg of protein) used for NH_2-terminal analysis did not catalyze formation of formamide from cyanide or hydrolysis of formamide to formate. Furthermore, stoichiometric conversion of cyanide into formate and ammonia was observed under both aerobic and anaerobic conditions in reaction mixtures of the purified enzyme which contained only NaCN (50 mM) in 0.1 M phosphate buffer at pH 7.5. It is highly unlikely that one or more enzymes present as contaminants in the purified enzyme (purity, >97%) are involved in the conversion of cyanide to formate and ammonia. Also, the action of many known cyanide-converting enzymes can be ruled out on theoretical grounds alone or because cosubstrates necessary for these reactions (15, 16) were not present in the assay mixture containing purified cyanidase. Formamide is not hydrolyzed by cyanidase, and we were unable to detect free formamide during hydrolysis of cyanide to formate. In conclusion, all our data are consistent with cyanide being hydrolyzed to formate and ammonia by a single enzyme. This does not, however, exclude the possibility that the reaction proceeds by, e.g., a two-step mechanism in which formamide occurs as an enzyme-bound intermediate which is not released during the catalytic cycle. Since cyanide is charged, in contrast to organic nitriles, we have, as mentioned above, named the cyanide-hydrolyzing enzyme from strain DF3 cyanidase in order to distinguish it from known nitrilases (EC 3.5.5.1). Nitrilases have not been reported to hydrolyze cyanide, and this may indicate that cyanidase should not be classified under EC 3.5.5.1 but should be given a new number.

There are several reports on microbial production of ammonia from cyanide (10, 14, 16, 26, 27, 30), but the enzymatic pathways involved have not been elucidated. Production of ammonia from cyanide may occur via a number of different enzymatic pathways (14–16) and is in itself, therefore, no proof of the involvement of a single enzyme. However, one recent report by White and coworkers (31) describes the isolation of a *Pseudomonas* sp. which appears to produce a cyanidase. This isolate required several transfers in cyanide-containing media to attain maximum activity.

The results described in this article and those published by White et al. (31) establish for the first time that bacteria carry out direct hydrolysis of cyanide to formate and ammonia. The data published so far (31) indicate that the enzyme produced by the *Pseudomonas* species is entirely different from that of strain DF3. It would therefore be interesting to compare the amino acid sequences and other characteristics of these two enzymes.

The cyanidase of strain DF3 may be useful for detoxification of cyanide wastes. Thus, whole cells of strain DF3 are able to function at very high cyanide concentrations and to hydrolyze cyanide to levels below 77 nM (~0.002 mg of CN^- per liter). Moreover, recent studies in our laboratory (unpublished data) have shown that cyanide hydrolysis by whole cells of strain DF3 is surprisingly resistant to inactivation by a number of organic nitriles, aliphatic and aromatic alcohols (e.g., ethanol, methanol, and phenol), and cyanide complexes of heavy metals (e.g., Cu, Ni, and Zn) occurring in many industrial waste streams. For example, intact cells of strain DF3 degraded 50 mM cyanide within 6 h in a

solution containing 50 mM acrylonitrile and 50 mM acrylamide in 0.1 M phosphate buffer (pH 7.5) when applied in a concentration of 2 mg (dry weight) of cells per ml. As a general feature, intact cells or freeze-dried cells of strain DF3 exhibited a much higher operational stability than the purified enzyme—a fact most likely due to the protective action of the plasma membrane and the cell wall.

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