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Bacterial Degradation of Cyanide and Its Metal Complexes under Alkaline Conditions

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A bacterial strain able to use cyanide as the sole nitrogen source under alkaline conditions has been isolated. The bacterium was classified as *Pseudomonas pseudoalcaligenes* by comparison of its 16S RNA gene sequence to those of existing strains and deposited in the Colección Española de Cultivos Tipo (Spanish Type Culture Collection) as strain CECT5344. Cyanide consumption is an assimilative process, since (i) bacterial growth was concomitant and proportional to cyanide degradation and (ii) the bacterium stoichiometrically converted cyanide into ammonium in the presence of L-methionine-D,L-sulfoximine, a glutamine synthetase inhibitor. The bacterium was able to grow in alkaline media, up to an initial pH of 11.5, and tolerated free cyanide in concentrations of up to 30 mM, which makes it a good candidate for the biological treatment of cyanide-contaminated residues. Both acetate and D,L-malate were suitable carbon sources for cyanotrophic growth, but no growth was detected in media with cyanide as the sole carbon source. In addition to cyanide, *P. pseudoalcaligenes* CECT5344 used other nitrogen sources, namely ammonium, nitrate, cyanate, cyanoacetamide, nitroferricyanide (nitroprusside), and a variety of cyanide-metal complexes. Cyanide and ammonium were assimilated simultaneously, whereas cyanide strongly inhibited nitrate and nitrite assimilation. Cyanase activity was induced during growth with cyanide or cyanate, but not with ammonium or nitrate as the nitrogen source. This result suggests that cyanate could be an intermediate in the cyanide degradation pathway, but alternative routes cannot be excluded.

Cyanide is a natural compound produced by many organisms, including bacteria, algae, fungi, and plants, and is bioproduced in some cases as a defensive metabolite (32) or with invasive purposes (19). Plants are the main source of cyanide in the biosphere because they cogenerate cyanide with ethylene (34) in addition to generating cyanoglycosides and cyanolipids. Moreover, cyanide has also been shown to be produced as part of active iron-cyanide complexes of catalytic proteins (39). Cyanide causes severe environmental problems when produced in high amounts by anthropogenic activities, such as mining and the electroplating industry. As an example, the jewelry industry located in the city of Cordova, Spain, produces an alkaline residue (pH $>$ 13) containing up to 20 g of free cyanide liter^{-1} (around 0.77 M) together with high amounts of heavy metals complexed with cyanide, thus making this effluent highly toxic and environmentally hazardous. An event showing the potential risk of cyanide occurred on 30 January 2000, when almost 100,000 m^3 of wastewater with a high concentration of cyanide was discharged into the Tisza River, a Danube tributary, causing a massive poisoning of the ecosystem.

Cyanide is highly toxic for most living organisms because it forms very stable complexes with transition metals that are essential for protein function, i.e., iron in cytochrome oxidase. Consequently, organisms growing in the presence of cyanide must have a cyanide-insensitive metabolism, such as the alternative oxidase described for plants (7) or the cytochrome *bd* (or cyanide-insensitive oxidase) in bacteria (24, 31, 40). The presence of cyanide in the environment causes an additional problem, the formation of extremely stable metal-cyanide complexes that make essential metals unavailable to the organisms. Therefore, bacterial proliferation in the presence of cyanide requires specific metal uptake systems. The strategy for iron uptake consists of the production of organic compounds, generically called siderophores, which strongly bind iron and are subsequently transported and assimilated (for reviews, see references 4 and 16). Finally, a cyanotrophic microorganism requires an assimilatory pathway able to convert cyanide into ammonium (for reviews, see references 12 and 38 and the references therein). To summarize, the biological assimilation of cyanide needs, at minimum, the concurrence of three separate processes, i.e., a cyanide resistance mechanism, a system for metal acquisition, and a cyanide assimilation pathway. Although all of these factors in conjunction with one another have never been taken into account, a number of microorganisms that are able to degrade cyanide and its metal complexes have been described to date (6, 12, 20, 22, 38).

From a chemical point of view, the biological treatment of industrial effluents contaminated with cyanide requires an alkaline pH in order to avoid the formation of the volatile HCN (pK_a = 9.2). In addition, since cyanide is known to react

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chemically with some keto groups (the Kiliani reaction), the use of glucose or similar C sources should be avoided. Previous works have reported microbial degradation of cyanide at neutral or acidic conditions (6, 38, 46). Strains adapted to survive in the presence of cyanide at alkaline pH but unable to degrade it have also been described (13). By contrast, references describing cyanide biodegradation at alkaline pHs are scarce. One of them refers to the fungus *Fusarium solani* (13), which uses cyanide as the sole nitrogen source. The bacterium *Burkholderia cepacia* is able to consume cyanide optimally at pH 10, but it needs glucose as a carbon source and is relatively sensitive to metal ions, such as iron and copper (1, 2). In this report, we report for the first time a bacterial isolate (*Pseudomonas pseudoalcaligenes* CECT5344) that is able to grow by using cyanide as the sole nitrogen source under alkaline conditions and acetate as the C source. The regulation of the process and the assimilation of alternative nitrogen sources are also described. In addition, this bacterium tolerates up to 30 mM of free cyanide and may also use several cyano-metal complexes, even in the presence of ammonium or nitrate. Therefore, this strain offers new perspectives in the bioremediation of industrial effluents contaminated with cyanide.

MATERIALS AND METHODS

Bacterial isolation. *P. pseudoalcaligenes* CECT5344 was isolated after an enrichment cultivation procedure. Basically, the medium used was the M9 minimal medium (30) prepared without ammonium and citrate, at pH 9.5, with 2 mM NaCN and 50 mM acetate as the sole added nitrogen and carbon sources, respectively. The medium was inoculated with sludge from the left margin of the Guadalquivir River downstream from the city of Córdoba and incubated in an Erlenmeyer flask at 30°C in a rotatory shaker. Cyanide was completely depleted after 2 weeks, and the process was repeated four times by reinoculation in fresh medium with 1% (vol/vol) of the previously grown culture. Samples of the enriched culture were plated on Luria-Bertani (LB) medium solidified with 1.8% Bacto agar (Difco), and individual colonies were purified and tested for axenic growth in liquid cultures with cyanide as the sole nitrogen source. Only one type of colony was able to assimilate cyanide, thus producing an axenic culture from which a single colony was selected, obtained in pure culture, and kept for further analysis.

Culture media. The bacterium was grown either in M9 minimal medium (without ammonium and citrate) adjusted to pH 9.5 or in LB medium on a rotatory shaker at 230 rpm and 30°C. Unless otherwise stated, 50 mM acetate was used as the carbon source. The appropriate nitrogen sources were added from sterilized stocks at the indicated concentrations. When the residue generated in the jewelry electroplating baths (hereafter called residue) was used as the nitrogen source, only the free cyanide was taken into account, because the method used routinely for cyanide determination detects only free cyanide. Moreover, the residue was used mainly to demonstrate the applicability of the biodegradation technology (see Chemicals section below).

For the detection of siderophores, the chrome azurol S agar medium was prepared as previously described (42). The plates with Prussian blue $\{Fe_4[Fe(CN)_6]_3\}$ were prepared by adding 0.75 mM soluble Prussian blue (Fluka) to M9 agar plates (pH 7) containing 50 mM acetate and, where indicated, 6 mM ammonium. The Prussian blue was added either to the whole plate or as top agar dissolved in 1% agar.

Taxonomic position and analysis of 16S rRNA gene sequence of *P. pseudoalcaligenes* CECT5344. Genomic DNA from the strain CECT5344 was obtained by using a Wizard kit (Promega) following the instructions of the manufacturer. The gene encoding the small ribosome subunit (16S rRNA) was entirely amplified by PCR with the universal eubacterial primers EUB-8F and EUB-1492R targeting positions 8 and 1492, respectively, of the *Escherichia coli* 16S rRNA numbering. PCR products were then cleaned by using a QIAGEN kit according to the manufacturer's protocol and sequenced in both directions by the Big Dye (Applied Biosystems) method.

Forward and reverse sequences thus obtained were aligned and manually checked to produce a high quality consensus sequence. After a BLAST analysis of the sequence, the isolate was tentatively identified as *P. pseudoalcaligenes* (99% similarity).

Analytical determinations. Bacterial growth was monitored by determining the absorbance at 600 nm. Nitrate, nitrite, and ammonium concentrations were determined as previously described (9). The ammonium concentration was also determined by using an ammonium-sensitive electrode. The free cyanide concentration was determined colorimetrically (5). The formamide concentration was calculated by the method described by Powell et al. (35) and modified by Schygulla-Banek (43). The protein concentration was determined by using a modified version of the Lowry method (44).

Cell dry weights were obtained by drying the pellets at 100°C to a constant weight, after centrifugation of the cultures.

Enzymatic activities. Nitrate reductase activity was determined as previously described (8).

Cyanase was assayed by the method described by Anderson (3).

Cyanide oxygenase was assayed according to the method of Fernández et al. (17), by monitoring the cyanide-dependent (0.1 to 2 mM) oxidation of NADH (0.2 mM) by cell extracts (up to 1 mg ml^{-1}). In addition to NADH, NADPH or H_4biopterin were also used. The activity was tested from pH 7 to 9 at intervals of 0.5 units by using the appropriate buffers (phosphate and Tris at 50 mM concentrations). Cyanide and ammonium were also assayed at the end of the experiments and compared to control test tubes containing boiled extracts.

Cyanidase (also called nitrilase and cyanide dihydratase) was assayed by measuring the ammonium formed from cyanide. The assay mixture contained, in a total volume of 5 ml, a 50 mM concentration of buffer (either phosphate or Tris, pH 7 to 9), 2 mM cyanide, and cell extract (up to 5 mg of protein). The same protocol was used to detect cyanide hydratase activity, but in this case formamide was measured instead of ammonium.

Formamide hydrolase was measured by monitoring the enzymatic conversion of formamide into ammonium as described above for cyanidase.

β-Cyanoalanine synthase was assayed as described by Dunnill and Fowden (14) with cysteine, serine, or *O*-acetyl-serine as the substrate.

Rhodanese activity was determined according to the method of Ray et al. (37).

Chemicals. The residue from the electroplating industry was kindly supplied by GEMASUR (Córdoba, Spain). The total cyanide concentration varies from 15 to 37 M, depending on the sample (47) whereas the free cyanide varies from 25 mM to 1 M. The difference between free and total cyanide is due to the presence of metals that complex most of the cyanide. The analysis of the residue used in this study revealed the presence of 0.76 M free cyanide (20 g $liter^{-1}$). The concentrations of the most abundant metals, estimated by atomic absorption, were 3.5 mM for Fe, 0.6 mM for Cu, 0.76 mM for Au, and 3.5 mM for V (B. Vallejo-Pecharromán and M. D. Luque de Castro, personal communication). The residue showed a pH higher than 13. When used as the nitrogen source, the residue was diluted in the culture medium to give the desired concentration of free cyanide (usually 2 mM, which corresponds to 2.63 ml of residue per liter of medium).

A 12.5 mM stock solution of $[Cu(CN)_4]^{-2}$ was prepared by mixing equal volumes of 100 mM KCN (sterilized by filtration) and 25 mM $CuSO_4$ (sterilized by autoclaving). When used as the nitrogen source, this solution was diluted in the culture medium to give the desired concentration.

The rest of the reagents were all of the maximal purity commercially available.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper, corresponding to the 16S RNA gene of *P. pseudoalcaligenes* CECT5344, has been annotated and deposited in the EMBL, DDBJ, and GenBank nucleotide sequence databases under the accession number AJ628163.

RESULTS AND DISCUSSION

Although biological removal of cyanide from industrial wastes is an attractive alternative to its chemical destruction, it has been applied only in a few cases, e.g., the Homestake mine (49). The oxidation numbers of C and N in cyanide are C(+II) and N(−III), that is, equivalent to CO (or formic acid) and NH_3, respectively. According to the current knowledge, all of the microorganisms able to assimilate cyanide can use it only as a nitrogen source, but not as the sole carbon source. By contrast, under anaerobic conditions, cyanide may be used as an alternative electron acceptor producing methane and ammonia, either in mixed (45) or axenic (25) cultures.

The main factor accounting for cyanide elimination in bio-

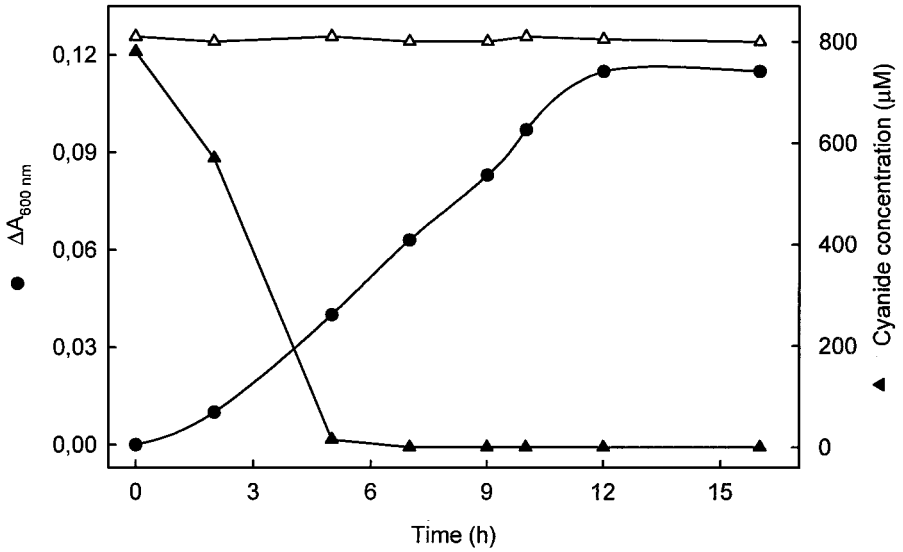

FIG. 1. Utilization of cyanide by *P. pseudoalcaligenes* CECT5344. Cells were precultured with 2 mM ammonium to the stationary phase ($A_{600} \approx 0.3$). At this point (time zero), the culture was separated into two flasks and 1 mM cyanide was added to one of them, whereas the other remained as a control without any additions. At the indicated times, the increments of cell growth of the culture growing with cyanide with respect to the control culture (●) and the cyanide concentration in the culture supernatant (▲) were measured. The concentration of cyanide in a noninoculated flask containing culture medium (△) was measured at the same times. Data are from a representative experiment.

degradation processes is the evaporation of HCN due to the neutral or acidic conditions of the medium (36). In addition, the formulation of the medium must be taken into account because cyanide can react with aldehydes and ketones (i.e., glucose), masking the biological degradation (Kiliani reaction). In this work, we report for the first time the degradation of cyanide by a bacterium in minimal medium without glucose and under alkaline conditions. The strain was isolated by enrichment cultivation in selective media at pH 9.5 with 2 mM NaCN as the sole nitrogen source as described in Materials and Methods. The bacterium was slightly curved, rod-shaped (0.4 by 1.5 μm), and gram negative. It was able to use ammonium, nitrate, nitrite, cyanoacetamide, nitroferricyanide, several amino acids (Ser, Thr, Ala, Met, Glu, and Gln), and some cyanide-metal complexes as nitrogen sources, in addition to cyanide. For carbon sources, the bacterium was able to use acetate, D,L-malate, and glucose. In all cases, metabolism was strictly aerobic. The bacterium does not contain detectable plasmids. In LB agar plates, the strain was resistant to the following antibiotics: tetracycline (10 μg ml^{-1}), ampicillin (100 μg ml^{-1}), chloramphenicol (10 μg ml^{-1}), and spectinomycin (200 μg ml^{-1}). By contrast, it was sensitive to kanamycin (25 μg ml^{-1}), streptomycin (200 μg ml^{-1}), and gentamicin (20 μg ml^{-1}). The optimum pH for growth, as deduced from the highest growth rate and shortest lag phase, was 9, with 50 mM acetate and 5 mM ammonium as the respective carbon and nitrogen sources. The bacterium has been classified as *P. pseudoalcaligenes* by comparison of the 16S RNA gene sequence with existing sequences and deposited in the Colección Española de Cultivos Tipo (Spanish Type Culture Collection) as strain CECT5344.

Cyanide degradation by *P. pseudoalcaligenes* CECT5344 seems to be an assimilative process since cyanide removal coincided with the exponential growth phase as well as with the maximal rate of oxygen consumption. This finding was further corroborated by using L-methionine-D,L-sulfoximine, which irreversibly inhibits ammonium assimilation at the level of the glutamine synthetase (10, 33). In the presence of L-methionine-D,L-sulfoximine, resting cells grown with cyanide catalyzed the stoichiometric conversion of cyanide into ammonium, which was accumulated in the culture medium (data not shown). By contrast, ammonium was not detected in the growth medium of untreated cultures.

P. pseudoalcaligenes CECT5344 tolerated an initial pH of up to 11.5 and a NaCN concentration of up to 30 mM. Nevertheless, due to the trapping of atmospheric CO_2, the pH of the medium becomes acidified during the experiments and some cyanide evaporates. To avoid this problem, the cyanide was added to cells precultured with a limiting amount of ammonium (2 mM). The addition of cyanide to ammonium-limited cultures has been used previously to induce enzymatic activities in *Pseudomonas fluorescens* (27, 29, 41). Under these conditions, the strain CECT5344 consumed 1 mM NaCN in 5 h (Fig. 1). Moreover, the cell growth was nearly the same with NaCN as with NH_4Cl, while it was higher with the residue (data not shown). Under these experimental conditions, neither cyanide (Fig. 1) nor ammonium (data not shown) evaporated from noninoculated control flasks. These results clearly show that cyanide can be fully degraded by this strain and suggest that the strain may also use cyano-metal complexes, according to the high quantity of heavy metal-cyanide complexes present in the residue from the jewelry industry, in addition to free cyanide. A further indication for the utilization of complexes was the appearance of a reddish precipitate at the end of the exponential growth phase, which probably corresponds to ferric iron. Therefore, the degradation of several metal complexes by *P. pseudoalcaligenes* CECT5344 was investigated. The strain was able to use as the sole nitrogen source

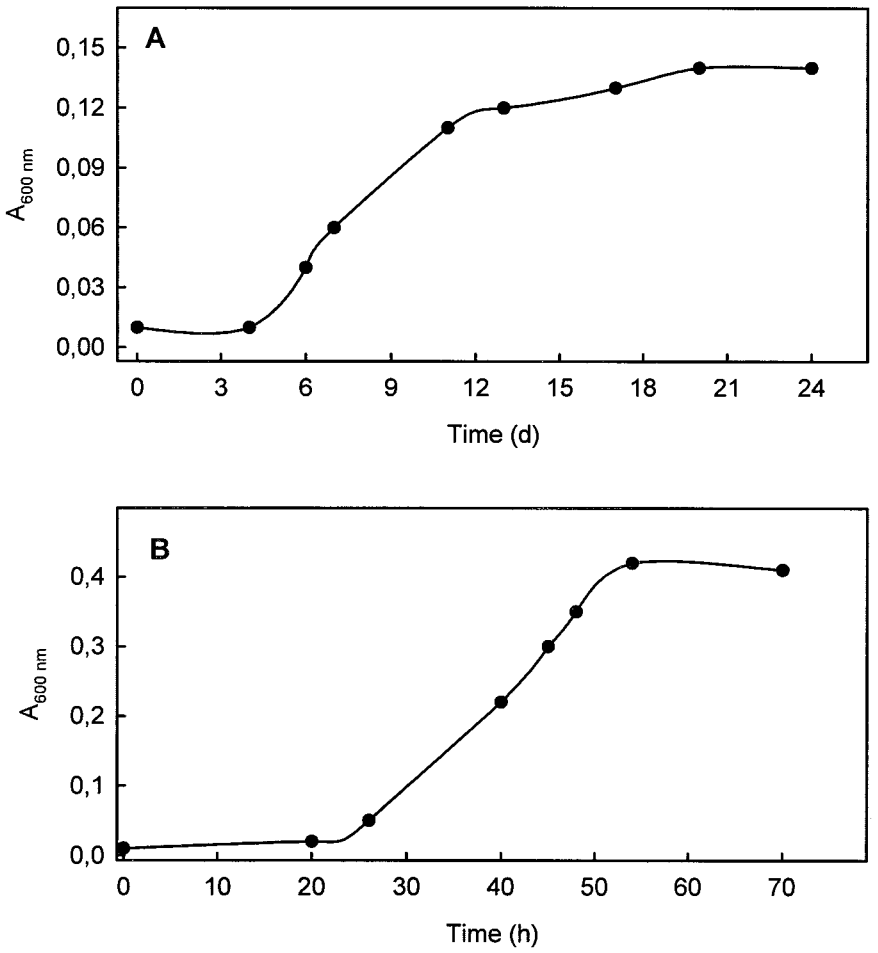

FIG. 2. Growth of *P. pseudoalcaligenes* CECT5344 with cyano-metal complexes as the sole nitrogen source. Cells were inoculated in media at pH 9.5 containing 1 mM potassium ferrocyanide (A) and 2 mM $K_2Cu(CN)_4$ (B) as the sole nitrogen sources. The cell growth was measured at the indicated times. No significant growth (less than 10%) was observed for cultures without cyanide. The experiment was repeated three times with similar results.

the cyanocomplexes of Fe(II) and Cu(II) at pH 9.5 (Fig. 2) and also at pH 7.5 in the presence of 200 μM 2,2′-bipyridyl, a strong iron chelator (data not shown). By contrast, the Zn(II) complex was poorly used at both pH values. *P. pseudoalcaligenes* CECT5344 was also able to use nitroprusside as the sole nitrogen source (data not shown). The production of siderophores was checked with plates with chrome azurol S agar (42), where the colonies formed a colorless halo due to the elimination of iron from the green complex. In addition, the bacteria grew on agar plates containing Prussian blue as the sole added iron source. The first microbial isolate found to grow on a metal-cyanide complex was *P. fluorescens* with $Ni(CN)_4^{2-}$ (41). The authors demonstrated that $Cu(CN)_4^{2-}$ is also a suitable nitrogen source for *P. fluorescens*, but to a lesser extent than the Ni complex. *P. pseudoalcaligenes* CECT5344 also used $K_2Cu(CN)_4$ as a nitrogen source (Fig. 2) but, as in the reference cited above, it was a poorer substrate than ammonia or free cyanide. The cyanide-metal complexes can be divided into weak acid-dissociable (WAD) and strong complexes. In WAD complexes, cyanide is readily released from the complexes when the pH is lowered to 4.5 to 6. Therefore, WAD refers to any free cyanide already present and cyanide released from nickel, zinc, copper, and cadmium complexes (but not from iron or cobalt complexes). WAD cyanide is generally considered to be the best current measure for assessing human and animal toxicity. The iron complexes are very stable and for this reason less toxic and more recalcitrant. In this sense, it was not surprising that $K_3Fe(CN)_6$ was the worst nitrogen source used by the CECT5344 strain. The stability of the complexes depends on the pH; they are, in general, more stable as the pH increases. Strikingly, *P. pseudoalcaligenes* CECT5344 degraded ferricyanide faster at pH 9.5 than at pH 7, in contrast to *F. solani* (6) and *P. fluorescens* (15) which use ferrocyanide only at pH 5 but not at alkaline pHs. Probably, the optimum pH for degrading metal-cyanide complexes is the result of a balance between the stability of the complex and the optimum pH for growth. Interestingly, an almost linear growth with cyano-metal complexes (Fig. 2) was observed, which suggests that some medium component is limiting or that some cellular component can't be synthesized under these circumstances. In any case, this bacterium is able to use both free cyanide and its metal complexes at alkaline pHs, thus providing

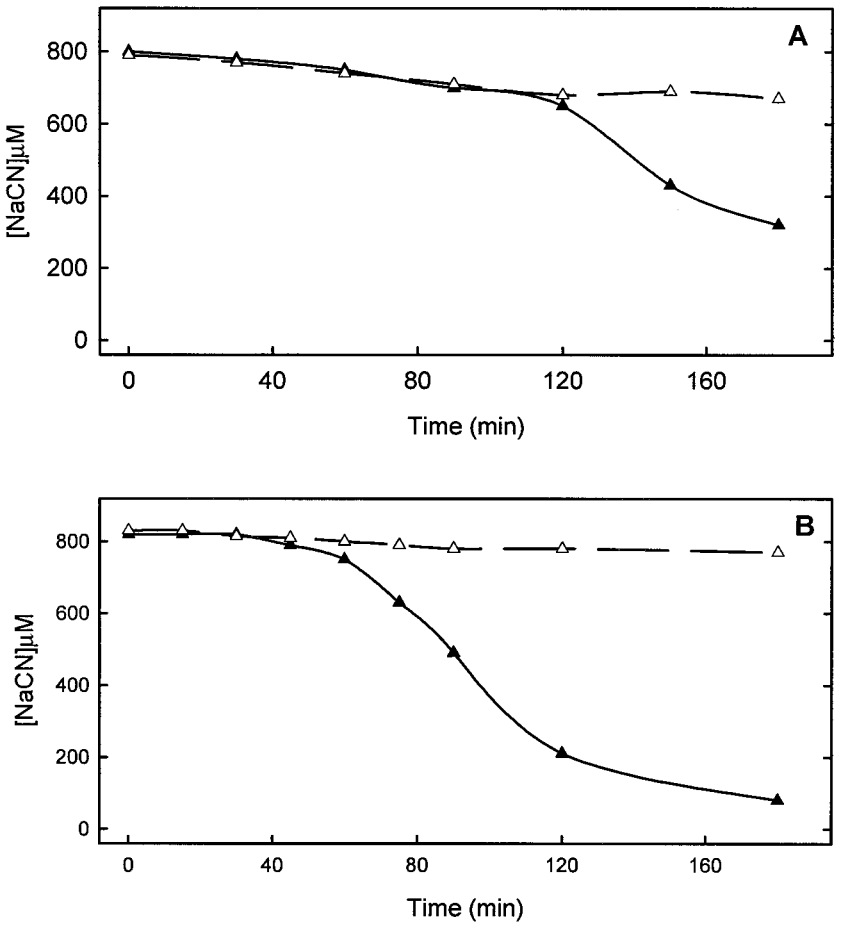

FIG. 3. Induction of the cyanide removal system in *P. pseudoalcaligenes* CECT5344. The cells were grown with 5 mM ammonium (A) and 4 mM cyanide (B) and harvested by centrifugation. After washing with nitrogen-free minimal medium, cells were resuspended in minimal medium containing 1 mM cyanide up to an A_{600} of 0.6 and placed in a rotatory shaker at 30°C. The cyanide concentrations in the culture supernatants were measured at the indicated times. In both cases, the open symbols and dashed lines correspond to the evolution of cyanide in noninoculated controls. The experiment was repeated three times with similar results.

a clear advantage since both cyanide volatilization and precipitation of its metal complexes are prevented. In addition, the tolerance of the bacterium to heavy metals is also a clear improvement over other strains that can degrade cyanide under alkaline conditions but are very sensitive to the presence of heavy metals (2).

In order to investigate the regulation and induction of the cyanide assimilation process in *P. pseudoalcaligenes* CECT5344, cyanide removal was checked in resting cell experiments with cells grown with several nitrogen sources. Cyanide-grown cells began to consume cyanide around 60 min before ammonium-grown cells (Fig. 3). Nitrate- and nitrite-grown cells behaved similarly to ammonium-grown cells (data not shown). These results suggest that the cyanide assimilation process is inducible. Curiously, the cyanide-grown cells needed a lag period to start cyanide consumption, unless they were resuspended in the same supernatant medium after centrifugation (data not shown). This result suggests that an extracellular metabolite may be necessary for cyanide removal. In this sense, both α-keto acids and siderophores have been described as putative components for cyanide assimilation by *P. fluorescens* NCIMB11764 (11, 27). Whether this is the case for *P. pseudoalcaligenes* CECT5344 deserves further investigation. Preliminary results show the accumulation of ketoacids, mainly 2-ketoglutarate, at the beginning of the exponential growth phase with cyanide as the sole nitrogen source.

Both the enhancement of biodegradation by second substrates and the inhibition of the process by alternative nutrients have been described widely in the literature. These effects are important not only regarding the applicability of the process, but also from a regulatory point of view. Therefore, the effect of alternative nitrogen sources on cyanide removal was investigated. To avoid cyanide volatilization, cells grown with a limiting amount of ammonium (2 mM) were used as mentioned above. Cyanide was consumed in the presence of either ammonium (Fig. 4A), nitrate (Fig. 4B), or nitrite (data not shown). Nevertheless, cyanide was not consumed by the bacterium in LB medium (data not shown). Ammonium is usually the preferred nitrogen source for bacteria. Consequently, ammonium (or some metabolite produced from it) usually exerts a negative regulatory effect on the assimilation of several nitrogen sources (9, 10). In this sense, the inhibition of cyanide

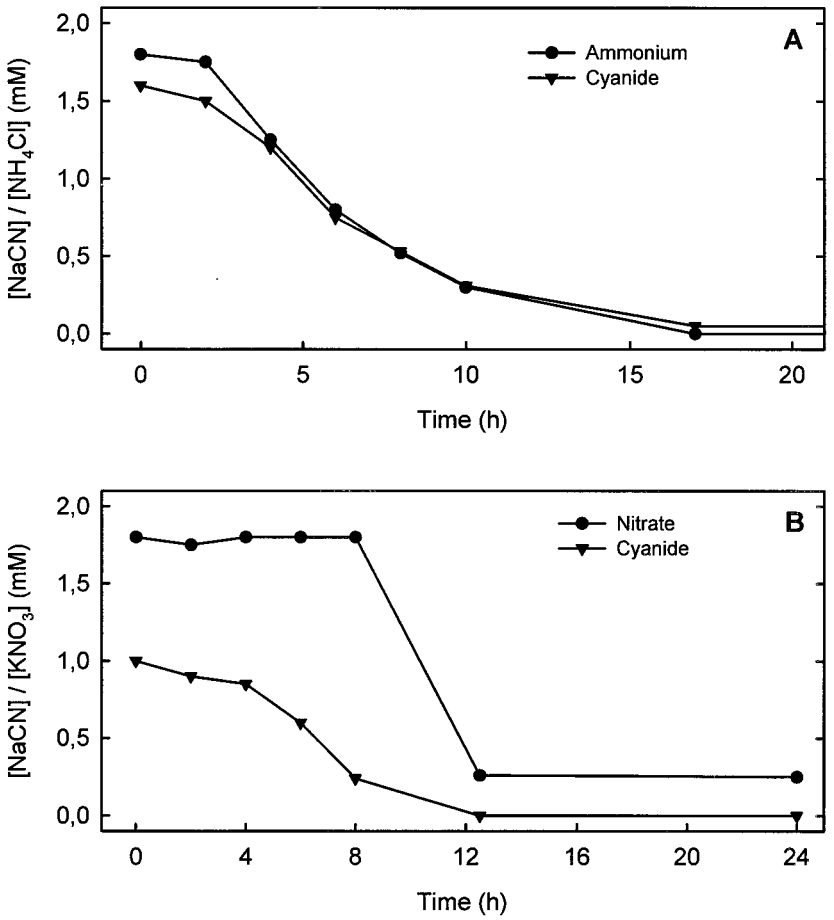

FIG. 4. Utilization of cyanide by *P. pseudoalcaligenes* CECT5344 in the presence of additional nitrogen sources. Cells were previously grown with a limiting amount of nitrogen (2 mM ammonium) with respect to the carbon source (70 and 50 mM acetate in panels A and B, respectively) in two separate flasks. After the complete ammonium consumption, cyanide was added to both of them, plus either ammonium (A) or nitrate (B). At the indicated times, aliquots of the cultures were centrifuged and the concentrations of the nitrogen sources was determined as indicated in Materials and Methods. The data are from a single experiment. Three independent experiments gave similar results.

assimilation by ammonium has been described in the literature and seems to be a general fact for cyanotrophic organisms (21, 22, 46). However, this is not the case for the CECT5344 strain, because the bacterium was able to assimilate both compounds simultaneously (Fig. 4A). Accordingly, the lag phase observed after the addition of cyanide to ammonium-growing cells has to be attributed to the need for the induction of other processes, i.e., the mechanism of resistance to cyanide, the acquisition of metals, or both. The inhibition of nitrate assimilation by cyanide has not been reported to date, whereas the in vitro inhibition of the assimilatory nitrate reductases by cyanide is well known. The nitrate reductase of this strain was also inhibited by cyanide in vitro (data not shown), giving an explanation for the inhibitory effect of cyanide on nitrate assimilation (Fig. 4B). Once more, and from the applicability point of view, *P. pseudoalcaligenes* CECT5344 seems to be superior to other cyanotrophic strains so far described because it can assimilate cyanide in the presence of ammonium or nitrate.

The enzymatic reactions for cyanide degradation can be categorized into four types: substitution/addition, hydrolysis, oxidation, and reduction (38). The hydrolytic pathways can be of two types; in one, ammonium forms directly by the addition of two molecules of water, whereas in the second, water is added sequentially with formamide as the intermediate. The generation of formamide from cyanide, catalyzed by cyanide hydratase activity, was first described for pathogenic fungi of cyanogenic crops (18). The addition of a second molecule of water catalyzed by an amidase leads to the formation of ammonium that may be used as the nitrogen source. This pathway is exclusive for fungi, whereas cyanide dihydratases have been described for both fungi and bacteria (23). *P. pseudoalcaligenes* CECT5344 was unable to use formamide as a nitrogen source. In addition, enzymatic activities catalyzing either the formation of formamide from cyanide, the hydrolysis of formamide into ammonium, or the direct conversion of cyanide into ammonium were not detected in cell extracts from cells grown with cyanide. Therefore, despite the fact that the bacterium was able to use some amides, such as cyanoacetamide, as a nitrogen source (data not shown), the existence of a hydrolytic pathway for the assimilation of cyanide in this strain seems to be unlikely.

Regarding to the substitution/addition pathways, the cata-

lyzed reaction of cyanide with cysteine, serine, *O*-acetyl-serine, or thiosulfate was undetectable.

An oxidative pathway was first proposed by Harris and Knowles (21), and later the incorporation of oxygen into the substrate was clearly demonstrated (48). Theoretically, the action of a monooxygenase can produce cyanate, which in turn is the substrate of the cyanase that catalyzes the formation of ammonium and CO_2 from cyanate and bicarbonate. Therefore, a pathway composed of two enzymes was originally proposed. The cyanide oxygenase, measured either by oxygen or NADH consumption, was hardly detected in cell extract of *P. pseudoalcaligenes* CECT5344 grown with cyanide. Only cell extracts obtained from cells treated with successive additions of 1 mM cyanide showed some cyanide-stimulated consumption of NADH (data not shown). Nevertheless, ammonia was never detected as a reaction product, as described for other bacteria (17, 21, 29), even in the presence of H_4biopterin as cofactor. By contrast, high levels of cyanase activity were measured with cells grown with cyanide or cyano-metal complexes as nitrogen sources (more than 400 U g^{-1} of protein). As this activity was not present in cells grown with ammonium or nitrate (data not shown), a pathway including cyanate as the intermediate can be proposed for the CECT5344 strain. As with *P. fluorescens* NCIMB11764 (26), the cyanase activity of CECT5344 was also induced by cyanate (data not shown). In contrast, the cyanase of *P. fluorescens* is repressed by ammonium and is not induced by cyanide. Further experiments demonstrated that cyanase activity of *P. fluorescens* is not essential for cyanotrophic growth (29). Unfortunately, the induction of the cyanase activity of *P. pseudoalcaligenes* CECT5344 was not exclusive for media containing cyanide, since the activity was also detected in cells grown with urea and even in nitrogen-free medium, although at lower levels (around 50 U g^{-1} of protein). Therefore, other routes for the assimilation of cyanide cannot be ruled out, as proposed for *P. fluorescens* NCIMB11764, which assimilates cyanide through several alternative routes (28, 29). Experiments are in progress to generate both random cyanide assimilation mutants and a site-directed mutant of the cyanase gene in order to elucidate the pathway used by this bacterium.

Finally, we note that *P. pseudoalcaligenes* CECT5344 is the first bacterium described to date that is able to degrade, in minimal-mineral medium without glucose, cyanide and cyano-metallic complexes under alkaline conditions. This capability, in combination with the ability to use cyanide in the presence of heavy metals, ammonium, or nitrate and the unusual resistance to cyanide, make this strain a very good candidate for the biotreatment of cyanurated residues.

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