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BIODEGRADATION

A Cyanide-Induced 3-Cyanoalanine Nitrilase in the Cyanide-Assimilating Bacterium *Pseudomonas pseudoalcaligenes* Strain CECT 5344

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ABSTRACT *Pseudomonas pseudoalcaligenes* CECT 5344 is a bacterium able to assimilate cyanide as a sole nitrogen source. Under this growth condition, a 3-cyanoalanine nitrilase enzymatic activity was induced. This activity was encoded by *nit4*, one of the four nitrilase genes detected in the genome of this bacterium, and its expression in *Escherichia coli* enabled the recombinant strain to fully assimilate 3-cyanoalanine. *P. pseudoalcaligenes* CECT 5344 showed a weak growth level with 3-cyanoalanine as the N source, unless KCN was also added. Moreover, a *nit4* knockout mutant of *P. pseudoalcaligenes* CECT 5344 became severely impaired in its ability to grow with 3-cyanoalanine and cyanide as nitrogen sources. The native enzyme expressed in *E. coli* was purified up to electrophoretic homogeneity and biochemically characterized. Nit4 seems to be specific for 3-cyanoalanine, and the amount of ammonium derived from the enzymatic activity doubled in the presence of exogenously added asparaginase activity, which demonstrated that the Nit4 enzyme had both 3-cyanoalanine nitrilase and hydratase activities. The *nit4* gene is located downstream of the cyanide resistance transcriptional unit containing *cio1* genes, whose expression levels are under the positive control of cyanide. Real-time PCR experiments revealed that *nit4* expression was also positively regulated by cyanide in both minimal and LB media. These results suggest that this gene cluster including *cio1* and *nit4* could be involved both in cyanide resistance and in its assimilation by *P. pseudoalcaligenes* CECT 5344.

IMPORTANCE Cyanide is a highly toxic molecule present in some industrial wastes due to its application in several manufacturing processes, such as gold mining and the electroplating industry. The biodegradation of cyanide from contaminated wastes could be an attractive alternative to physicochemical treatment. *P. pseudoalcaligenes* CECT 5344 is a bacterial strain able to assimilate cyanide under alkaline conditions, thus avoiding its volatilization as HCN. This paper describes and characterizes an enzyme (Nit4) induced by cyanide that is probably involved in cyanide assimilation. The biochemical characterization of Nit4 provides a segment for building a cyanide assimilation pathway in *P. pseudoalcaligenes*. This information could be useful for understanding, and hopefully improving, the mechanisms involved in bacterial cyanide biodegradation and its application in the treatment of cyanide-containing wastes.

KEYWORDS biodegradation, cyanide, nitrilase

Nitriles are organic compounds harboring a cyano group (R-CN). In this sense, cyanide can be considered the simplest nitrile (H-CN). Since cyanide is a very reactive molecule, nitriles can be formed after the addition of cyanide to different molecules, such as unsaturated organic substrates, imines, or keto groups, for example.

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Another possibility for the formation of nitriles is the substitution of the appropriate leaving group after the nucleophilic attack of cyanide. A clear example of this mechanism is the replacement of the -SH group in the cysteine molecule by cyanide in the reaction catalyzed by the β-cyanoalanine synthase (1). Cyanogenesis and cyanide metabolism are especially important in plants, involving many genes (2).

The addition of cyanide to organic molecules and the further formation of amino acids from radiolabeled cyanide were reported several decades ago, either in bacteria (3, 4), fungi (5), or plants (6–8). One example was the finding that cyanide can be incorporated into a carbon skeleton as β-cyanoalanine (4, 7, 9). The corresponding enzyme, β-cyanoalanine synthase (CAS), was purified from lupine seedlings and shown to be a pyridoxal-dependent enzyme that catalyzes the formation of β-cyanoalanine and sulfide from cyanide and L-cysteine (1). Since cyanide was not recognized at that time as a common metabolite in green tissue metabolism, it was somehow troubling that enzymes for cyanide metabolism were present in most plants. The discovery of cyanide as the equimolecular coproduct in the biosynthesis of the plant hormone ethylene (10) provides a reason for the ubiquitous presence of cyanide metabolism pathways in plants (11). Bacterial degradation of cyanide though β-cyanoalanine was also proposed several decades ago (12).

Many plants may produce extra cyanide as a defensive mechanism, thus protecting them against pathogens and predators (13–15). Therefore, herbivore insects have developed several mechanisms to avoid cyanogenic glycoside toxicity (16). Conversely, plant growth can be inhibited by cyanide produced by some cyanogenic bacteria (17). In a recent publication, it was demonstrated how mite and lepidopteran species that thrive on plants defended by cyanogenic glucosides can survive by expressing a gene (the β-cyanoalanine synthase [CAS] gene), probably transferred from bacteria, that protects arthropods from cyanide poisoning (18). This mechanism can be regarded in the context of the molecular evolution of xenobiotic metabolism and resistance in mites (19). The CAS activity found in the gut of some herbivore insects can be the sum of different isoenzymes (20). In addition to its role as a weapon, the cyanide molecule has been proposed to play a regulatory role in plants under certain circumstances (21). In this sense, it has been demonstrated that CAS activity is essential to maintain a low level of cyanide for proper root hair development in *Arabidopsis thaliana* (22).

Not only cyanide but nitriles might also be toxic by themselves by inhibiting specific enzymes (23–25). In this sense, β-cyanoalanine is a neurotoxin that has also been found in the defensive droplets secreted by some insects (26). Since nitrilases are the enzymes that hydrolyze nitriles, their function could be avoiding the toxicity of nitriles, including cyanide. In this sense, the quantity of nitrilase activity could limit the cyanide detoxification pathway, as demonstrated by the fact that increasing β-cyanoalanine nitrilase activity expands cyanide tolerance in *Arabidopsis* (27).

The nitrilase family of enzymes is one of the 13 branches in which the members of the carbon-nitrogen hydrolase superfamily have been classified (28). In plants, there are at least 4 types of genes encoding nitrilases (*NIT1* to *NIT4*), with NIT4 being ubiquitous and highly specific for 3-cyanoalanine (29). Therefore, this enzyme, in conjunction with the β-cyanoalanine synthase, has been proposed to play a role in cyanide detoxification in higher plants (30). The enzyme was shown to have both nitrilase (EC 3.5.5.4) and hydratase (EC 4.2.1.65) activities. The overexpression of a gene homologous to *nit4* (*pinA*) in *A. thaliana* resulted in increased root elongation in the absence of exogenous 3-cyanoalanine, suggesting that this nitrile may have a significant role in root physiology and root development (31). In general, nitrilases seem to play important roles in plant and bacterial metabolism and also in the plant-bacterium interaction (30, 32).

We coined the term cyanotrophs for organisms with the ability to grow on cyanide supplied as the sole nitrogen source (33), although cyanide-assimilating bacteria have been reported at least since the mid-20th century (3, 34–37). *Pseudomonas pseudoalcaligenes* CECT 5344 was isolated by enrichment cultivation based on its ability to assimilate cyanide under alkaline conditions (33). The sequencing of its genome

revealed the presence of an unusually high number of nitrilase genes (four) in comparison to other *Pseudomonadaceae* isolates (38). Only one of them (*nitC*) has been previously studied, and it was found to be important for cyanide assimilation (39), although the cyanide assimilation pathway was not elucidated, since the physiological substrate of NitC remains unknown. *nitC* corresponds to *nit3* in the paper describing the presence of four nitrilases in this bacterial strain (38). Here, we demonstrate that Nit4 from *Pseudomonas pseudoalcaligenes* CECT 5344 is a β-cyanoalanine nitrilase specifically induced by cyanide. Although CAS activity was not detected in this strain (33), the biochemical characterization of the enzyme as well as the induction pattern and synteny of *nit4* reported in this paper suggests that *nit4* may be part of an operon implicated in both cyanide resistance and assimilation.

RESULTS

3-Cyanoalanine nitrilase activity from *P. pseudoalcaligenes*. A putative nitrilase gene homologous to the gene encoding the 3-cyanoalanine nitrilase described in plants (*nit4*) was detected among the four putative nitrilase genes in the genome of *P. pseudoalcaligenes* CECT 5344 (38). Moreover, *P. pseudoalcaligenes* CECT 5344 has been reported to be able to assimilate 3-cyanoalanine (40). Since there are not published antecedents concerning either the assimilation pathway of 3-cyanoalanine in this strain or its role in cyanide assimilation, the presence of a putative 3-cyanoalanine nitrilase activity was studied. For that, cells were grown either in LB medium or in M9 minimal medium with ammonium or cyanide as the sole nitrogen source. The maximum 3-cyanoalanine nitrilase activity was observed in cell extracts from LB-grown cells (7 ± 2 mU/mg of protein), followed by cyanide-grown cells (6 ± 2 mU/mg of protein) and ammonium-grown cells (2 ± 1 mU/mg of protein). This apparent activity could not be true nitrilase activity, because measuring ammonium formation from 3-cyanoalanine by using cell extract cannot distinguish between the addition of two molecules of water in a single enzymatic step (nitrilase activity) and the consecutive action of hydratase plus amidase activity (Fig. 1A). In order to discriminate between these two possibilities, cell extract from cells grown with cyanide or ammonium was subjected to anion-exchange chromatography (Fig. 1B). The result was that true nitrilase activity could be detected only in cells grown with cyanide as the nitrogen source (Fig. 1Bb). No nitrilase activity was found in ammonium-grown cells (Fig. 1Ba) or in LB-grown cells (not shown). In contrast, asparaginase activity appeared in the same fraction in both ammonium- and cyanide-grown cells (Fig. 1B), as well as in LB-grown cells (not shown), thus suggesting that the asparaginase is constitutively expressed in this bacterium in all these culture media.

The nitrilase enzyme was shown to be very labile, because the activity disappeared from the cell extracts after 1 week whether they were frozen at −20°C, refrigerated at 4°C, or maintained at room temperature. Therefore, several protecting agents commonly used in sample preparation were used, namely, EDTA, 2-mercaptoethanol, glycerol, dithiothreitol (DTT), glutathione, and cOmplete protease inhibitor cocktail (Roche). The protease inhibitor cocktail clearly protected the enzyme, especially at short times (<1 day), and glycerol (5% [vol/vol]) was very useful for protecting the frozen enzyme for long periods of time. Consequently, the buffer in which the cells were broken was supplemented with the protease inhibitor cocktail, whereas this buffer as well as the rest of the buffers employed in further chromatographic steps contained 5% glycerol.

Nit4 expression and purification from *Escherichia coli*. The publication of the draft genome sequence of *P. pseudoalcaligenes* (38) allowed the design of specific primers for cloning the *nit4* gene into the expression vector pET-11d, as indicated in Materials and Methods. The new plasmid, named pETcan, allows the conditional expression of the gene under the control of the T7 RNA polymerase. The plasmid was electroporated into *E. coli* BL21, and the transformants were able to use 3-cyanoalanine as the sole nitrogen source when the expression of the gene was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) (Fig. 2). Moreover, the Nit4 enzyme expressed in *E. coli* eluted at the same fractions

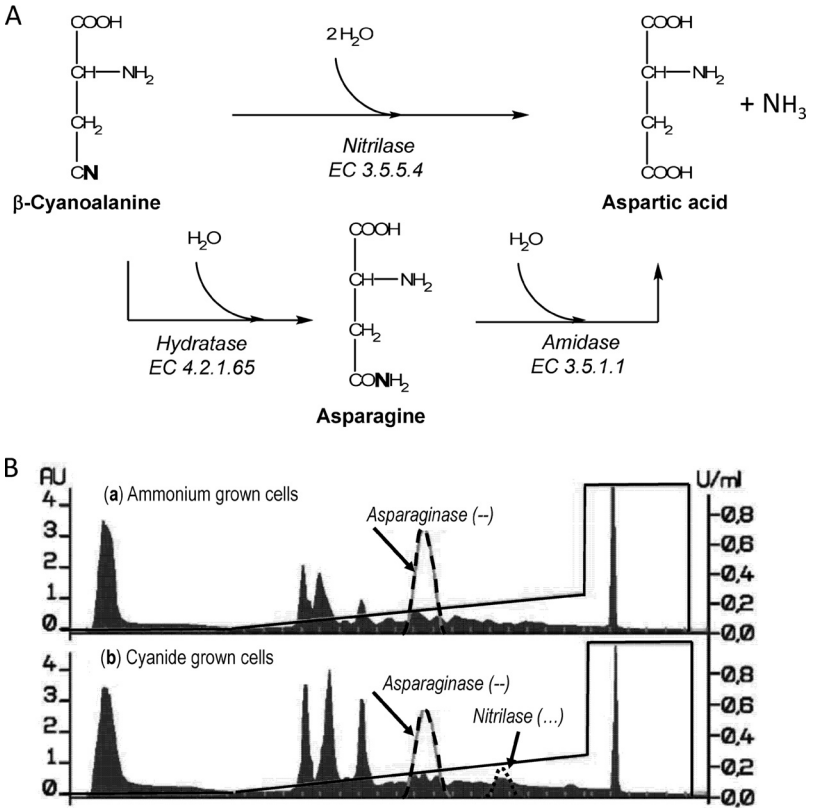

FIG 1 (A) Schematic representation of the possible pathways generating ammonium from 3-cyanoalanine. The top arrow indicates the direct of addition of two molecules of water rendering aspartic acid plus ammonium in a reaction catalyzed by the nitrilase (EC 3.5.5.4). Alternatively, ammonium can be released by the consecutive action of a hydratase (EC 4.2.1.65) and the corresponding amidase (asparaginase, EC 3.5.1.1). (B) Anion-exchange chromatography of cell extract of *P. pseudoalcaligenes* CECT 5344 R1 grown in M9 minimal medium with ammonium (a) or cyanide (b) as the sole nitrogen source. The concentrations of proteins that were eluted by applying a linear gradient, as indicated in Materials and Methods (solid line), were recorded by measuring the absorbance at 280 nm (arbitrary units [AU], represented as the black shape of the chromatogram). The asparaginase (dashed line) and nitrilase (dotted line) activities were measured in every fraction and represented as relative activity.

after anion-exchange chromatography (Fig. 1B) and had the same kinetic properties as the enzyme induced by cyanide in *P. pseudoalcaligenes* (not shown). These results indicate that *nit4* is the same 3-cyanoalanine nitrilase gene induced by cyanide in *P. pseudoalcaligenes* CECT 5344 and provides a native overexpressed enzyme for protein purification according to the protocol indicated in Materials and Methods, which is summarized in Table 1. The lability of the enzyme observed in the cell extracts of *P. pseudoalcaligenes* (see above) becomes reflected in the poor yield obtained at the end of the process, mainly due to the last two purification steps. Using this protocol, the enzyme has been purified up to homogeneity, as deduced from electrophoretic studies (Fig. 3). As observed in Fig. 3, a single protein band was detected after the 6th purification step, corresponding to a mass of approximately 35 kDa, which is consistent with the theoretical molecular mass derived from the sequence of the BN5_1912 gene of 35,379.712 Da. Nevertheless, the apparent molecular mass of the native Nit4 protein from *P. pseudoalcaligenes* CECT 5344 R1 determined by the use of gel filtration was approximately 440 kDa, suggesting a dodecameric quaternary structure. Gel filtration chromatography was also employed to calculate the Stokes radius of the native protein, which was approximately 57.8 Å. The optimum pH of the enzymatic activity was 8, and its optimum temperature was 45°C, although the enzyme was relatively stable at higher temperatures. The enzyme remained fully active after incubating it at 55°C for 10 min, but it maintained some activity up to 65°C. The presence or absence of added asparaginase did not change significantly the apparent K_m of the

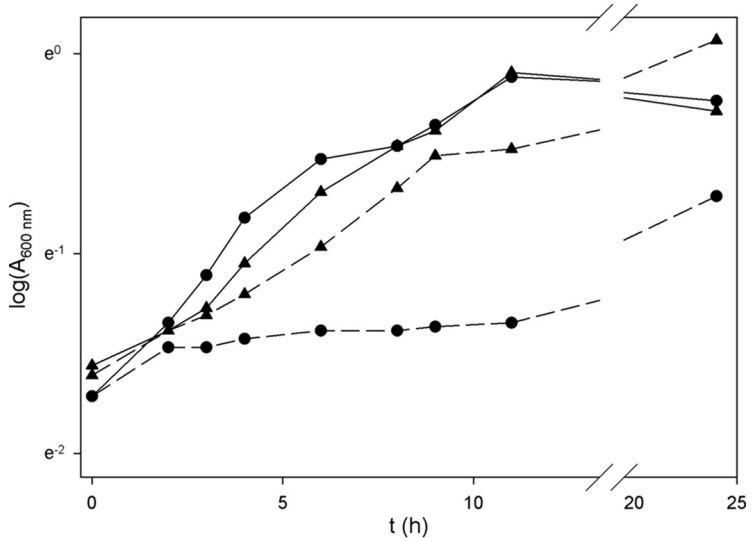

FIG 2 Expression in *E. coli* of the *nit4* gene from *P. pseudoalcaligenes* CECT 5344 R1. Growth curves of *E. coli* BL21 containing the empty cloning plasmid pET-11b (●) or the plasmid with a translational fusion of *nit4* from *P. pseudoalcaligenes* CECT 5344 R1 (▲) in M9 minimal medium with 4 mM ammonium chloride (solid lines) or 2 mM 3-cyanoalanine (dashed lines) as the sole nitrogen sources. The culture medium was supplemented with IPTG (0.5 mM) to induce the expression of *nit4*. The experiments were carried out in triplicate, but the figure shows the results of a representative experiment. t, time.

enzyme for 3-cyanoalanine, giving values of 1.37 ± 0.42 mM and 1.35 ± 0.51 mM, respectively.

The substrate specificity of the enzyme was studied by measuring ammonium formation under optimal conditions with different nitriles as the substrate, at 5 mM final concentration. The compounds tested were cyanide, acetonitrile, benzonitrile, 3-indole acetonitrile, lactonitrile, 3-hydroxypropionitrile, glycolic acid nitrile, and acetone cyanohydrin. No enzymatic activity was observed with any of these compounds under optimal conditions, thus revealing a very narrow substrate specificity.

The quantity of ammonium formed in the reaction catalyzed by the nitrilase activity reached a maximum that was approximately half the amount of 3-cyanoalanine initially added as the substrate (Fig. 4A). Since the nitrilase enzyme did not have asparaginase activity (Fig. 4B), the putative accumulation of asparagine was investigated. On the other hand, the partially purified asparaginase activity from *P. pseudoalcaligenes*, which did not have nitrilase activity (Fig. 4A), catalyzed the stoichiometric production of ammonium from asparagine (Fig. 4B). Finally, almost-stoichiometric amounts of ammonium from 3-cyanoalanine were detected only when both nitrilase and asparaginase activities were present together in the assay mixture (Fig. 4A). This experiment was repeated with different proportions of the two enzymes and different initial concentrations of 3-cyanoalanine. The result was that the amount of ammonium formed from 3-cyanoalalanine at the end of the experiment when both enzymes were added was

TABLE 1 Purification of Nit4 from *P. pseudoalcaligenes* CECT 5344 R1 expressed in *E. coli* BL21 pETcan

Purification step	Vol (ml)	Protein (mg)	Nit4 activity		Yield (%)	Purification factor
			Total (U)	Specific (U/mg)		
Crude extract	12	357.0	1,368	3.83	100.0	1.0
Heating (55°C)	11	275.0	1,397	5.08	102.1	1.3
Mono Q 10/10	14	56.0	863	15.41	63.1	4.0
$(NH_4)_2SO_4$ Frac[a]	1	19.0	799	42.05	58.4	11.0
Gel filtration	5	2.0	87	43.50	6.4	11.4
MonoQ 5/50	1	0.3	16	53.33	1.2	13.9

[a]Frac, fractionation.

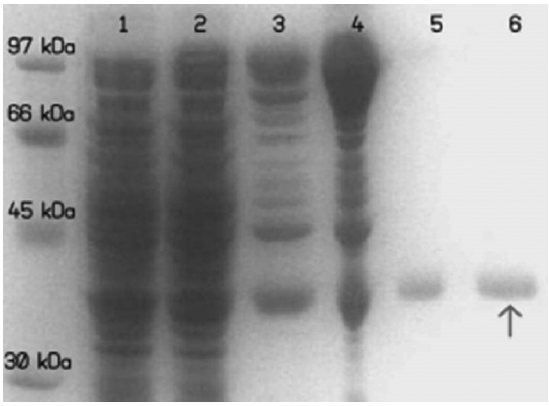

FIG 3 SDS-PAGE gel, stained with Coomassie brilliant blue, of the protein fractions obtained in the consecutive steps used for the purification of Nit4 (Table 1). Lane 1, crude extract; lane 2, heated extract followed by centrifugation; lane 3, active fractions from Mono Q 10/10; lane 4, ammonium sulfate fractionation; lane 5, after gel filtration; lane 6, active fractions after Mono Q 5/50. The unnumbered left column contains the protein standards. The arrow indicates the position of Nit4.

double the amount of ammonium formed in the assay mixtures containing nitrilase activity only.

Targeted disruption of *nit4* gene in *P. pseudoalcaligenes* CECT 5344. As stated before, the strain CECT 5344 was described to be able to use 3-cyanoalanine as the sole nitrogen source (40). Then, in order to check the putative role of Nit4 in the assimilation of cyanide and this nitrile, the phenotype of a mutant in the corresponding gene was studied. The mutant exhibited almost the same growth rate as the wild-type (WT) strain with ammonium or cyanide as the sole nitrogen source (Fig. 5A and B). In contrast, the growth rate of the mutant was lower than that of the WT strain with 3-cyanoalanine as the sole nitrogen source (Fig. 5C), a difference that increased drastically in the presence of KCN (Fig. 5D).

Regulation of *nit4* expression in *P. pseudoalcaligenes*. Since Nit4 activity was detected only in minimal medium with cyanide as a nitrogen source (Fig. 1), and the assimilation of 3-cyanolanine was also enhanced by cyanide (Fig. 5D), the expression of the corresponding gene was studied by quantitative PCR, as indicated in Materials and Methods. The result, as summarized in Fig. 6A, was that the expression of *nit4* was clearly induced by cyanide either in minimal medium (5-fold increase with respect to ammonium-grown cells) or LB medium (15-fold increase after the addition of 1 mM cyanide with respect to LB-grown cells).

DISCUSSION

Nitrilases are enzymes catalyzing the hydrolysis of nitriles to the corresponding carboxylic acids plus ammonia (41). These enzymes have received extensive attention because they were shown to have several biotechnological applications in the so-called green chemistry (42), including uses in synthetic processes, such as industrial production of acrylamide and nicotinamide (43). β-Cyanoalanine is an endogenous plant compound that was identified in *Vicia sativa* as a possible factor causing human neurolathyrism (24).

In *Pseudomonas pseudoalcaligenes* CECT 5344, it was described that 2-oxoacids chemically react with free-cyanide-producing 2-hydroxynitriles, which can be theoretically converted into ammonium by either a nitrilase or a nitrile hydratase-amidase system (40). Nevertheless, the precise pathway for cyanide assimilation is unknown. The expression of *nit4* in *E. coli* allowed the recombinant strain to fully assimilate 3-cyanoalanine as a nitrogen source (Fig. 2). During the first 12 h, it was evident that only the strain expressing *nit4* was able to assimilate 3-cyanoalanine, which is consistent with the detection of 3-cyanoalanine nitrilase activity in cell extracts of the same cells. Nevertheless, at longer incubation times (up to 25 h), both the strain with the

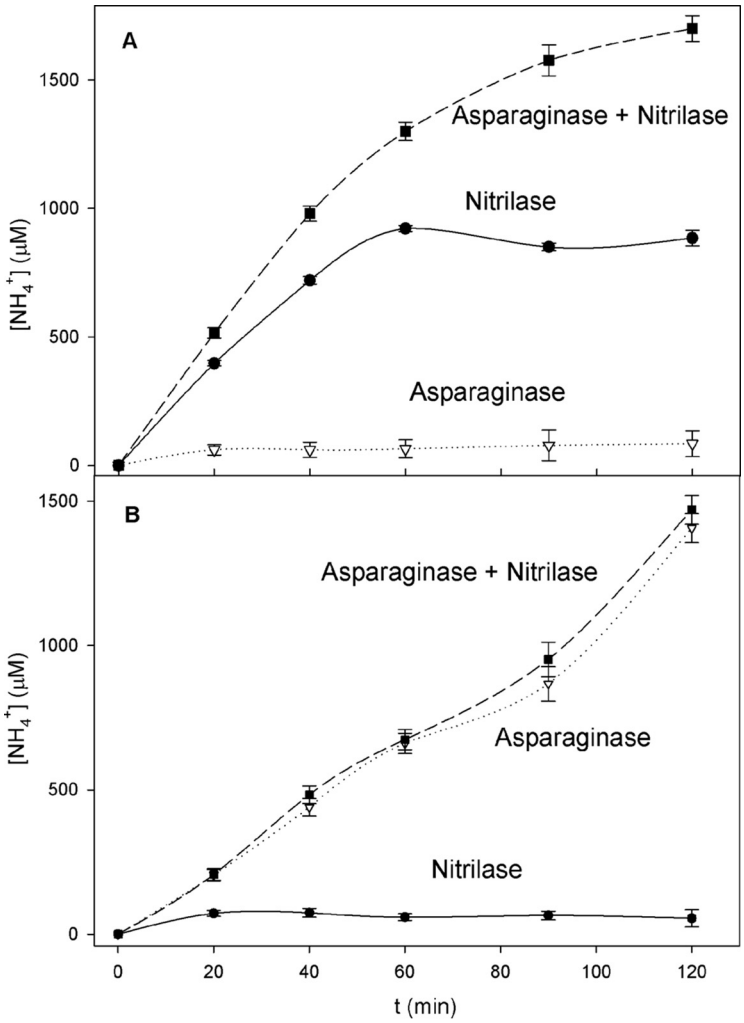

FIG 4 Ammonium formation from 1.5 mM 3-cyanoalanine (A) or asparagine (B) in reactions catalyzed by 12 mU asparaginase (▽, dotted lines), 20 mU nitrilase (●, solid lines), or mixtures of both enzymes (■, dashed lines), from *P. pseudoalcaligenes* CECT 5344 R1. Data are the averages of the results from three independent experiments. The activity was measured as indicated in Materials and Methods.

cloned gene and that with the hollow plasmid exhibited a further increase in cell density. These results are consistent with the utilization of the α-amino group of 3-cyanoalanine by both strains, whereas only the strain expressing *nit4* is able to use the nitrogen derived from the nitrile. Overall, the conclusion from the data in Fig. 2 is that the expression of Nit4 in *E. coli* results in a gain of function with respect to the parental strain in fully assimilating 3-cyanoalanine.

Purification of the enzyme was possible after its expression in *E. coli*, once it was confirmed that this enzyme had the same molecular properties as the enzyme induced by cyanide in *P. pseudoalcaligenes* CECT 5344. The heat treatment of the extract was a very effective purification step of the enzyme expressed in *E. coli* (Table 1 and Fig. 3). In contrast, this treatment had little effect when used in the background of *Pseudomonas pseudoalcaligenes* CECT 5344 (not shown). There is no obvious reason for this apparent thermotolerance, but it is curious to observe how the two enzymes purified from *P. pseudoalcaligenes* CECT 5344 until now, cyanase (44) and β-cyanoalanine nitrilase (this work), are thermoresistant. Moreover, fumarase and aconitase enzymes are also thermoresistant up to 60 degrees (not shown). Although it is expected to find a thermoresistant nitrilase in thermophilic bacteria (45), some mesophilic bacteria harboring thermoresistant nitrilases have been also described (46).

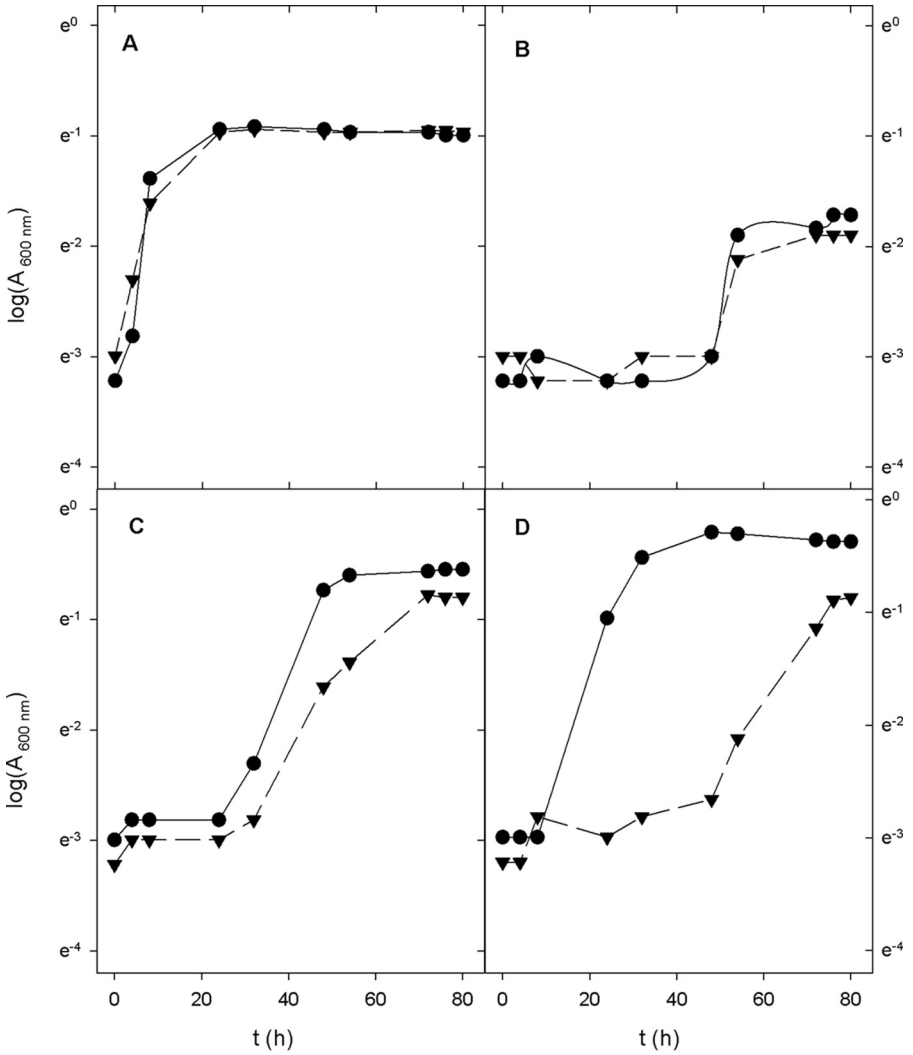

FIG 5 Phenotype of the Δ*nit4* mutant of *Pseudomonas pseudoalcaligenes* CECT 5344 R1. The WT strain (●, solid lines) and the *nit4* deletion mutant (▲, dashed lines) were cultured in M9 minimal medium with acetate as carbon source and the following nitrogen sources: 2 mM ammonium chloride (A), 2 mM potassium cyanide (B), 2 mM 3 cyanoalanine (C), or 2 mM 3 cyanoalanine plus 1 mM cyanide (D). At the indicated times, cell growth was measured as the absorbance of the culture at 600 nm (A_{600}). The experiments were carried out in triplicate, but the figure shows the results of a representative experiment.

The K_m of the nitrilase from *P. pseudoalcaligenes* CECT 5344 is similar to the K_m of Nit4 from *Arabidopsis thaliana* (0.74 mM (29) but about 1 order of magnitude lower than that of the enzyme purified from *Pseudomonas* sp. 13 (47), although the two bacterial enzymes have very similar molecular weights (monomer) and similar optimum pHs and temperatures (47). Nevertheless, the oligomeric structure of the protein purified from *P. pseudoalcaligenes* CECT 5344 (dodecamer) is different from that described in *Pseudomonas* sp. strain 13 (M_r of approximately 1,000,000 [47]) but similar to other bacterial nitrilases (48).

The substrate specificity of the enzyme seems to be very narrow. In fact, the only substrate for this enzyme was 3-cyanoalanine among the nitriles tested in this work. The same results were obtained for the similar proteins purified from either higher plants (29) or bacteria (47). Nevertheless, since the reaction was followed by the formation of ammonium, the hydration of some of the tested nitriles cannot be completely discarded.

The stoichiometry of the reaction as well as the substrate specificity of the nitrilases varies depending on the biological source (41). In *P. pseudoalcaligenes* CECT 5344, the

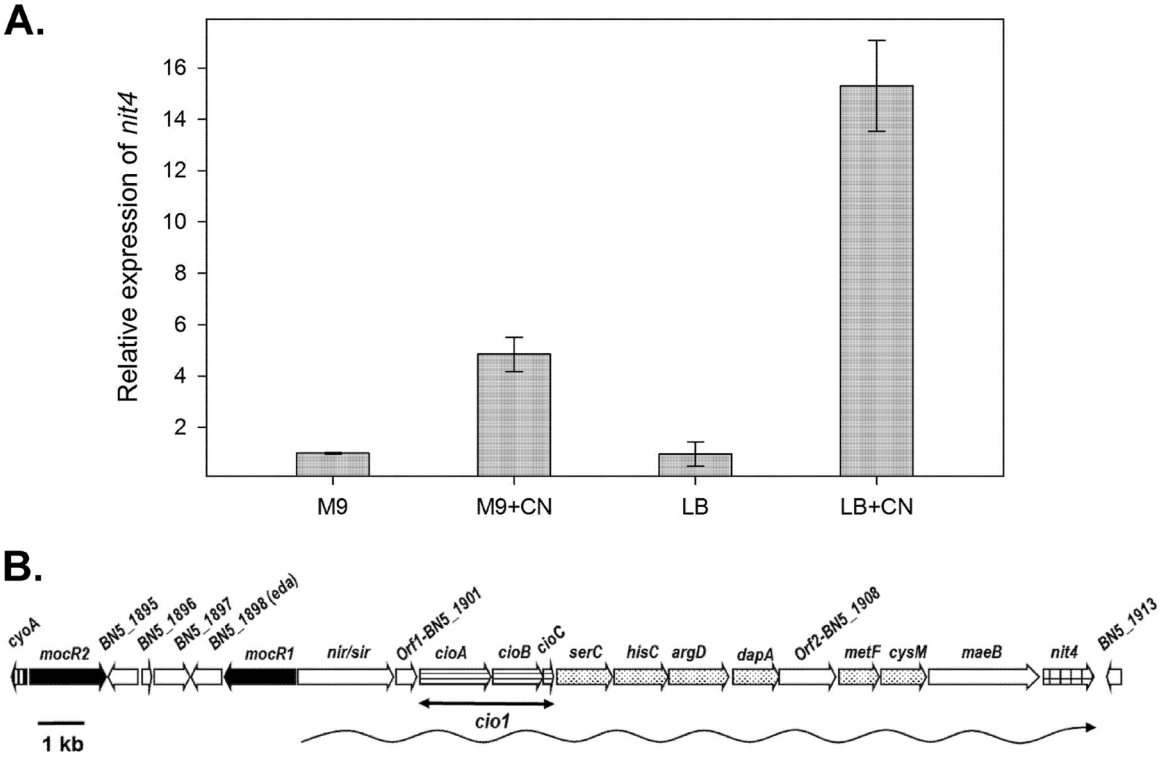

FIG 6 (A) Effect of cyanide on the expression of *nit4* in minimal and complex (LB) medium. The relative expression of *nit4* in minimal and LB media was calculated as described in Materials and Methods. (B) Synteny of the *nit4* gene in the context of the *cio1* operon. The genes are represented as arrows, the lengths of which are approximately to scale with the reference bar. Black arrows represent putative regulatory genes, striped arrows are genes for terminal oxidases (*cio*, horizontally striped; *cyo*, vertically striped), white arrows are genes of unknown function, and dotted arrows are genes related to amino acid metabolism. *cyoA* (BN5_1893), putative pseudogene of *cyoA*; *mocR2* (BN5_1894), *gntR*-like putative regulator; BN5_1995-97, genes for hypothetical proteins; BN5_1898, 2-dehydro-3-deoxyphosphogluconate aldolase/4-hydroxy-2-oxoglutarate aldolase gene (*eda*); *mocR1*, *gntR*/*mocR*-family transcriptional regulator gene (BN5_1899); *cysI*, putative nitrite/sulfite reductase gene (*nir*/*sir*); *cioA*-*cioC*, cyanide insensitive oxidase-*cio1* operon; *serC*, phosphoserine aminotransferase gene; *hisC*, histidinol-phosphate aminotransferase gene; *argD*, acetylornithine delta-aminotransferase gene; *dapA*, dihydrodipicolinate synthase gene; BN5_1908, gene for major facilitator superfamily (MFS)-like protein; *metF*, methylenetetrahydrofolate reductase gene; *cysM*, cysteine synthase B gene; *maeB*, NADP-dependent malic enzyme gene; *nit4*, 3-cyanoalanine nitrilase gene.

amount of ammonium produced in the presence of both enzymes is double the amount of ammonium derived from the action of the nitrilase alone (Fig. 4A). Since this result was obtained with different initial concentrations of 3-cyanoalanine and is independent of the putative experimental errors determining the concentration of the nitrile, it can be concluded that the stoichiometry of the reaction catalyzed by the nitrilase from *Pseudomonas pseudoalcaligenes* CECT534 is, per 2 mol 3-cyanoalanine transformed, 1 mol asparagine, 1 mol aspartate, and 1 mol ammonium produced. Taking into account the water molecules, the reaction can be written as 2 (3-cyanoalanine) + 3 (H_2O) → 1 (aspartate) + 1 (asparagine) + 1 (NH_4^+). This result is somewhat similar to the 2:1 or 1.36:1 ratio obtained for the nitrile hydratase/nitrilase described in plants and other bacterial cyanoalanine nitrilases (29, 41, 47). In any case, it is remarkable that this enzyme does not accept as a substrate a compound that is assumed to be a reaction intermediate (i.e., asparagine).

From a physiological point of view, this result can be interpreted as the enzyme having an assimilatory role, since the products of the reaction are two proteinogenic amino acids plus ammonium. Conversely, the action of a typical nitrilase would produce only aspartic acid, with the reverse process for producing the proteinogenic amino acid asparagine being much more expensive from an energetic point of view. The regulation of the expression of this gene agrees with this hypothesis, since the enzymatic activity becomes expressed under cyanotrophic conditions. In fact, this bacterium assimilates 3-cyanoalanine much more efficiently in the presence of cyanide (Fig. 5D). The maximum growth reached by the wild-type strain with 2 mM 3-cyanoalanine as the nitrogen

source was almost double that with the same concentration of ammonium, thus suggesting that both the α-amino and the β-cyano nitrogen groups can be used as a nitrogen source by this strain. On the other hand, the observed reduced growth of the Δ*nit4* mutant with 3-cyanoalanine as the nitrogen source could be due to the utilization of the α-amino group (Fig. 5D). The same argument can be applied to interpret the differences in cell growth with 3-cyanoalanine as a nitrogen source for *E. coli* expressing *nit4* in comparison to the same strain transformed with the empty cloning plasmid (Fig. 2).

Pseudomonas sp. 13 also assimilates 3-cyanoalanine, and this compound is the inducer of the nitrilase activity (47). In contrast, it has been reported that in *Pseudomonas pseudoalcaligenes* CECT 5344 3-cyanoalanine does not induce the cyanide assimilation pathway (40), and here we demonstrate that the inducer of the *nit4* gene is clearly cyanide (Fig. 1B and 6A) and that 3-cyanoalanine is much more efficiently assimilated in the presence of cyanide (Fig. 5).

Previous studies have shown that cyanide induces the expression of the *cio1* operon (cyanide insensitive terminal oxidase of the respiratory chain) in *Pseudomonas pseudoalcaligenes* CECT 5344 (49). Sequencing of the genome of this strain (38) has revealed that the *cio1* genes are clustered with genes related to amino acid metabolism as well as the *nit4* gene itself. The synteny of nitrilase genes may give clues for understanding the evolution of the protein family (50). As far as we know, bacterial homologues to *nit4* have not been reported until now in the context described here. Therefore, it is tempting to propose that both cyanide resistance (*cio1*) and metabolism of amino acids, including 3-cyanoalanine nitrilase (up to *nit4*), that are coregulated by cyanide reside in the same operon and thus would be cotranscribed (Fig. 6B). Also remarkable is the presence of two putative regulatory genes belonging to the *gntR* family (*mocR* subfamily) just upstream and, with the closer one, in the opposite direction of transcription from *cio1*, an archetypical organization of bacterial operons. The roles of *mocR1* and *mocR2* in the regulation of the expression of *cio1* are not completely clear at this moment, but homologous sequences are in synteny with genes for terminal oxidases *cio* (cyanide insensitive oxidase) and *cyo* (bo_3-type quinol oxidase) in other bacterial strains (not shown). Notwithstanding, the *cyoA* sequence at the far left of the *cio1* cluster in *P. pseudoalcaligenes* (Fig. 6B) is a pseudogene, and so this bacterium lacks a complete *cyo* gene cluster. Most of the genes between *cio1* and *nit4* have paralogous counterparts in other loci of the genome, thus reinforcing the idea that they are not involved in the intermediary amino acid metabolism but in the metabolism of cyanide. Since the formation of 3-cyanoalanine has not been described in this bacterium, the product of these genes may well contribute to the regeneration of 3-cyanoalanine from aspartic acid and asparagine, which are the products of the nitrilase, probably with serine and cysteine as intermediates. This hypothesis, if proved, should be added to the different cyanide assimilation pathways proposed in this and other *Pseudomonas* strains (39, 51, 52).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. A spontaneous rifampin-resistant mutant (40 μg/ml) of *Pseudomonas pseudoalcaligenes* CECT 5344 (33) was used throughout the study, named *P. pseudoalcaligenes* CECT 5344 R1. The bacterium was grown as previously described (33), either in M9 minimal medium (without ammonium and citrate) adjusted to pH 8.5 or in LB medium on a rotary shaker at 230 rpm and 30°C. Unless otherwise stated, 40 mM acetate was used as the carbon source. The appropriate nitrogen sources (cyanide and/or ammonium and/or 3-cyanoalanine) were added from sterilized stocks at the indicated concentrations.

E. coli XL1-Blue (Stratagene) and *E. coli* BL21, which were used for general cloning purposes and for the heterologous expression of Nit4, respectively, were cultured in LB or M9 medium at 37°C and 230 rpm on a rotary shaker. Where indicated, the media were supplemented with ampicillin (100 μg/ml) and gentamicin (40 μg/ml) from sterile concentrated solutions.

Analytical determinations. Bacterial growth was monitored by following the optical density of the culture (absorbance) at 600 nm. Ammonium and cyanide concentrations were routinely determined colorimetrically using the Nessler assay (53) and the pyridine barbituric acid method (54), respectively. When a higher sensitivity for the determination of ammonium was needed, its concentration was determined by using the phenol-hypochlorite method (55).

TABLE 2 Primers utilized in this work

Description	Restriction enzyme	Primer name	Sequence (5′→3′)[a]
Primers for amplifying and cloning native *nit4*	NcoI	nit-n	TAG**CCATGG**CAAATAATGAGACT
	BamHI	nit-b	GTC**GGATCC**TGCCCAGGGAA
Primers for amplifying fragment used to mutate *nit4*	XbaI	15637U21	GC**TCTAGA**CGCTAGAACTGAT
	XbaI	17458L21	CG**TCTAGA**TTTGCTGGCCAGT
Primers for studying expression of *nit4* by qPCR		21	GCGCTCCCACAGTCGATGCT
		22	TCTCCGGTCCTTGCGTCTTG
Primers for iPCR using pK3can as template	BamH1	17032U21	GC**GGATCC**ACAGGCAGCCACT
	BamH1	16242L21	AC**GGATCC**GGAAACACGACGA
Primers amplifying the *aacC1*-Gm resistance cassette plus BamHI flanking sequences from pMS255	EcoRI	gent-upper	CCCTGAG**GAATTC**GAGCTCGG
	BamH1	gent-lower	CCCGG**GGATCC**TCTAGAGTCG
Primers amplifying the 16S RNA gene		G	CGGCGGACGGGTGAGTAATG
		H	CGGATCGTCGCCTTGGTGAG

[a]The sequence of the introduced target sequence is shown in bold.

Protein concentration was determined according to the method of Lowry et al. (56).

Cloning of *nit4* from *P. pseudoalcaligenes* CECT 5344 R1. Briefly, the strategy was as follows. The *nit4* gene was amplified by PCR using genomic DNA as the template and the nit-n and nit-b primers (Table 2). The resulting fragment was subjected to digestion with NcoI and BamHI and ligated into the expression vector pET-11d (Novagen), which was previously digested with the same restriction enzymes. The resulting plasmid (pET3Can) was electroporated into *E. coli* BL21 electrocompetent cells and the putative transformants selected in LB plates containing ampicillin.

Construction of an *nit4*-deficient mutant. To construct an *nit4*-deficient mutant, first, a 1.8-kb-long DNA region was amplified by PCR with primers 15637U21 and 17458L21 (Table 2) using genomic DNA from *P. pseudoalcaligenes* CECT 5344 R1 as the template. This fragment, which contains 480 bp upstream and 365 bp downstream of *nit4*, was cloned into the pK18mob plasmid by means of the XbaI restriction sites created by the primers. The new plasmid, called pK3can, was used as the template for an inverse PCR (iPCR) by using 17032L21 and 16242U21 primers (Table 2). The amplified fragment was identical to pK3can but lacked a 748-bp-long DNA central fragment of *nit4*. The new BamH1 restriction sites introduced by the primers allowed the cloning of the *aacC1* cassette conferring resistance to gentamicin (Gm) with pMS255 (see below). The resulting plasmid, called pKΔ3can, was electroporated into electrocompetent cells of *E. coli* XL1-Blue. The plasmid, which replicates in *E. coli* but not in *Pseudomonas*, was characterized and finally electroporated into *P. pseudoalcaligenes* CECT 5344 R1 electrocompetent cells prepared as described elsewhere (49). The mutants were selected in LB medium plates supplemented with gentamicin. Since the suicide plasmid (pK18mob) contains a kanamycin resistance gene, the absence of kanamycin resistance in the mutant was indicative of a double recombination event replacing the central part of *nit4* with the *aacC1* cassette. This fact was confirmed by Southern hybridization of EcoRV-digested genomic DNA from WT and Nit4 mutant strains against *nit4* and *aacC1* digoxigenin-labeled probes (not shown).

The *aacC1* Gm resistance cassette was amplified by PCR from the plasmid pMS255 (57). The primers were designed to introduce the desired restriction sites for further cloning requirements (Table 2).

Expression and purification of Nit4. The Nit4 enzyme was purified from *E. coli* BL21/pETCan, which contains a translational fusion of the *nit4* gene from *P. pseudoalcaligenes* CECT 5344 R1 in the pET-11d expression vector. *E. coli* pETCan cells were cultured overnight in 100 ml of LB medium supplemented with ampicillin (100 μg/ml) at 37°C and 230 rpm on a rotary shaker. Next day, 25 ml of this culture was used to inoculate 2 liters of LB medium, supplemented with IPTG (0.5 mM final concentration), and incubated for 5 h under the same conditions. The cells were collected by centrifugation and washed two times with 1/10 volumes of the original culture volume of 50 mM Tris-HCl (pH 8), and finally the cell pellets were resuspended in the same buffer containing 5% glycerol and cOmplete protease inhibitor cocktail, as recommended by the supplier (Roche). The cells were disrupted by two passages through a French pressure cell operated at 130 MPa and the cell debris removed by centrifugation at 18,000 $\times$ *g* for 15 min. The resulting supernatants (cell extracts) were heated at 55°C for 5 min, cooled in ice for 10 min, and finally centrifuged at 70,000 $\times$ *g* for 1 h in order to remove the precipitated proteins. The resulting supernatant was loaded onto an anion-exchange chromatography column (Mono Q 10/10 GL; Amersham Biosciences) equilibrated in the same buffer employed to prepare the cell extract (50 mM Tris-HCl [pH 8] with 5% glycerol) at a flow rate of 5 ml/min. After elution of the unbound proteins with 2 column volumes of buffer, the bound proteins were eluted by applying 20 column volumes of a linear gradient from 0 to 0.5 M NaCl in the same buffer and at the same flow rate. The chromatography experiments were carried out at 4°C in an ÄKTApurifier 10, thus allowing the continuous measurement of the concentration of proteins in the effluent (absorbance at 280 nm) and its fractionation in aliquots of 2 ml. The fractions containing nitrilase activity were pooled separately and subjected to ammonium

sulfate fractionation by the stepwise addition of the salt up to 40% saturation. After being gently stirred for 20 min, the suspension was centrifuged at 20,000 × *g* for 30 min. The supernatant was discarded and the resulting pellet suspended in a minimal volume of 50 mM Tris-HCl (pH 8) buffer supplemented with 5% glycerol. Up to 100 μl of this fraction was directly loaded onto Superdex 200 10/300 GL (General Electric) and isocratically eluted with 50 mM Tris-HCl (pH 8) supplemented with 5% glycerol at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected for further measurement of nitrilase activity. The column was previously calibrated with the gel filtration calibration kits from GE Healthcare, according to the manufacturer's instructions, thus allowing the determination of the apparent molecular weight of the native enzyme as well as its Stokes radius (R_{st}) (58). The molecular weight (M_r) and Stokes radius of the proteins used for calibrating the column were as follows: thyroglobulin, 669,000 and 85 Å; ferritin, 440,000 and 61 Å; catalase, 232,000 and 52.2 Å; aldolase, 158,000 and 48.1 Å; albumin, 67,000 and 30.5 Å; ovalbumin, 43,000 and 30.5 Å; chymotrypsinogen A, 25,000 and 20.9 Å; and RNase A, 13,700 and 16.4 Å. The void volume of the column (V_0) was estimated as the elution volume of dextran blue. The R^2 values for the linear regression representing K_{av} [$K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the sample and V_0 and V_t are the void volume and the total volume of the column, respectively] versus $\log_{MW}$ and $(-\log_{Kav})^{1/2}$ versus R_{st} of the calibrating curves were 0.989 and 0.97, respectively. The MW and R_{st} of the nitrilase were calculated by substituting its experimental K_{av} value obtained under the same conditions in the equation of the linearized calibration curves, constructed as indicated above. Using the purification protocol, the central fractions of the gel filtration chromatography were pooled and loaded into an anion-exchange column (Mono Q 5/50 GL) at a flow rate of 1 ml/min and eluted using the same gradient as used in the first anion-exchange chromatography, except that the linear gradient (20 column volumes) was from 0.1 to 0.4 M NaCl.

To test the purity of the fractions as well as the molecular weights of the subunits, the fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (59).

Chromatographic separation of asparaginase and nitrilase activities from *P. pseudoalcaligenes* CECT 5344 R1. The cells were grown in 2 liters of M9 minimal medium with cyanide or ammonium as the sole nitrogen source until mid-log-growth phase. The same protocol described above for the purification of Nit4 from *E. coli* BL21/pETCan was used, up to the first chromatographic step (Mono Q 10/10 GL). The fractions having asparaginase activity from *P. pseudoalcaligenes* CECT 5344 R1 were separated and pooled and stored at 4°C until needed for determining the stoichiometry of the reaction catalyzed by the nitrilase activity.

Nitrilase and asparaginase activity assays. Routinely, both nitrilase (EC 3.5.5.4) and asparaginase (EC 3.5.1.1) activities were assayed by measuring the quantity of ammonium formed after 30 min of incubation at 40°C in 50 mM Tris-HCl buffer with 10 mM substrate concentration and the appropriate amount of protein cell extract. One unit of activity is defined as the amount of enzyme producing 1 μmol ammonium per minute under assay conditions. For the kinetic characterization of the nitrilase activity, aliquots of the reaction mixture were taken over time and the ammonium concentration calculated in triplicate according to the Solorzano protocol (55). The initial velocity for each concentration of substrate was estimated in the linear part of the curve and used to calculate the K_m by means of the double-reciprocal plots (Lineweaver-Burk plots [60]). The K_m of the nitrilase for 3-cyanoalanine was also calculated, as explained above, in the presence of saturating amounts of asparaginase. In both cases, the K_m was calculated in triplicate. The time course of the reaction was also applied to determine the stoichiometry of the reaction catalyzed by the nitrilase activity, but adding to the assay mixture partially purified asparaginase from *P. pseudoalcaligenes* CECT 5344. Since the presence of ammonium obviously interferes in the determination of the enzymatic activity, protein extracts obtained by ammonium sulfate fractionation were dialyzed by means of PD-10 molecular exclusion columns (Pharmacia Biotech) or, alternatively, by ultrafiltration (Ultrafree-0.5; Millipore).

The optimum pH for the nitrilase was determined by measuring the activity in reaction mixtures containing 50 mM different buffers in intervals of 0.5 pH units. The buffers were potassium phosphate (6.5 to 7.5), Tris-HCl (7 to 9), and sodium carbonate (9.5 to 11).

The optimum temperature was obtained by carrying out the reaction under optimal conditions in tubes submerged in a water-thermostatic bath at the desired temperature. For determining its thermal stability, aliquots of the enzyme were incubated for 10 min at different temperatures (20°C to 70°C range) and cooled in ice for 5 min, and after centrifugation at 10,000 × *g* for elimination of precipitated proteins, the activities were measured under optimal conditions.

Quantitative expression of *nit4*. Cells were grown in LB medium up to an absorbance at 600 nm of approximately 0.3 unit. At this point, a volume of culture containing cells giving a final optical density of 3 units/ml (approximately 10 ml) was centrifuged for collecting the corresponding cells. The rest of the culture was treated with 2 mM KCN for 2 h and the cells collected by centrifugation. For minimal medium expression experiments, the cells were cultured with potassium acetate as carbon source and a limiting amount of nitrogen (2 mM NH_4Cl) up to the stationary phase (approximately 0.4 unit of absorbance at 600 nm). The culture was divided in two halves and incubated with 2 mM in KCN or with 2 mM NH_4Cl for two additional hours. At this point, a volume of each culture corresponding to an optical density of 3 units/ml was centrifuged.

The total RNA was extracted from the cells by using the Aurum total RNA minikit (Bio-Rad), as recommended by the manufacturer. The RNA samples were treated with the Turbo DNA-free kit (Ambion), frozen at −20°C for 2 h, and stored at −80°C until its use. Total RNA was retrotranscribed by means of the iScript cDNA synthesis kit (Bio-Rad), running in parallel control experiments without retrotranscriptase.

The relative expression of *nit4* was estimated with respect to the expression of the 16S RNA gene in each sample, but taking into account the efficiency of the reaction for each pair of primers (61). The primers used for *nit4* and 16S RNA genes are indicated in Table 2. The efficiency of each pair of primers was calculated by running the quantitative PCR (qPCR) with serial dilutions of the corresponding DNA and the relative expression calculated as previously described (62).

Accession number(s). The genome sequence of *P. pseudoalcaligenes* CECT 5344 can be found on the NCBI Web page under GenBank accession no. HG916826.1.

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