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Characterization of the Nit6803 nitrilase homolog from the cyanotroph *Pseudomonas fluorescens* NCIMB 11764

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ABSTRACT

We report the purification and characterization of a nitrilase (E.C. 3.5.5.1) (Nit11764) essential for the assimilation of cyanide as the sole nitrogen source by the cyanotroph, *Pseudomonas fluorescens* NCIMB 11764. Nit11764, is a member of a family of homologous proteins (*nitrile*_sll0784) for which the genes typically reside in a conserved seven-gene cluster known as Nit1C. The physical properties and substrate specificity of Nit11764 resemble those of Nit6803, the current reference protein for the family, and the only true nitrilase that has been crystallized. The substrate binding pocket of the two enzymes places the substrate in direct proximity to the active site nucleophile (C160) and conserved catalytic triad (Glu44, Lys126). The two enzymes exhibit a similar substrate profile, however, for Nit11764, cinnamonitrile, was found to be an even better substrate than fumaronitrile the best substrate previously identified for Nit6803. A higher affinity for cinnamonitrile (*K*m 1.27 mM) compared to fumaronitrile (*K*m 8.57 mM) is consistent with docking studies predicting a more favorable interaction with hydrophobic residues lining the binding pocket. By comparison, 3,4-dimethoxycinnamonitrile was a poorer substrate the substituted methoxyl groups apparently hindering entry into the binding pocket. *in situ* ^{1}H NMR studies revealed that only one of the two nitrile substituents in the dinitrile, fumaronitrile, was attacked yielding *trans*-3-cyanoacrylate (plus ammonia) as a product. The essentiality of Nit11764 for cyanotrophy remains uncertain given that cyanide itself is a poor substrate and the catalytic efficiencies for even the best of nitrile substrates ($\sim 5 \times 10^{3}$ M^{-1} s^{-1}) is less than stellar.

1. Introduction

Nitrilase (aminohydrolases) (E.C. 3.5.5.1) enzymes catalyze the direct hydrolysis of nitrile substrates to ammonia and the corresponding carboxylate (eq. 1). They represent a subgroup of enzymes in the large nitrilase superfamily (SUPFAM SSF56317) that consists of 13 separate branches, but only one of these (Branch 1) contains enzymes that actually catalyze nitrile hydrolysis [1–3]. By comparison, enzymes in Branches 2–9 catalyze reactions involving the hydrolysis of amides (amidases), carbamates (carbamylases) or perform the amidase reaction in reverse (*N*-acyltransferases) [1]. Branch 10 enzymes are unique in having originally been associated with tumor-suppressor activity in mice and humans [4]. More recently they have also been shown to have amidase activity towards toxic metabolic products generated during transamination such a α-ketoglutaramate and deaminated glutathione [5–7]. The remaining three branches of the superfamily are more poorly defined but are recognized by nitrilase-containing domains.

$$RCN + 2H_2O \rightarrow NH_3 + RCO_2H \quad (1)$$

Branch 1 enzymes have received considerable attention over the years because of their use as biocatalysts (the chemoenzymatic production of gabapentin [8], and atorvastatin (Lipitor^{R}) [9] being examples). Nitrile-hydrolyzing enzymes (aminohydrolases) are found in all domains of life and display some common properties. For example, most if not all consist of a ~40 kDa subunit that oligomerizes into a large structure containing as many as 22 subunits. The native structures assume an ααββ sandwich polypeptide arrangement and form large filament like particles with a twisted helical structure [2,10,11]. Details related to the helical twisted nature of these enzyme has received considerable attention of late because of its demonstrated importance in determining substrate specificity [12]. In the absence of available crystal structures the recent application of cryoEM has proven itself useful for

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these studies [13]. The one exception to this is Nit6803 (pdb id, 3uwy) from the cyanobacterium, *Synechocystis* sp. strain PCC6803, whose crystal structure has been determined [14] and its quaternary structure shown to assume a low twist helical arrangement.

Nit6803 is unique in that it represents one of a cluster of about 300 homologous proteins (*nitrile_sll0784*) for which the encoding genes typically reside in a conserved gene cluster known as Nit1C. The arrangement of genes in Nit1C, which form an operon, are illustrated in Fig. 1. Gene annotations are as follows: *nitB*, hypothetical, *nitC*, nitrilase, *nitD*, radical S-adenosyl-methionine enzyme, *nitE*, *N*-acetyltransferase (*nitE*), *nitF*, AIR synthetase, *nitG* hypothetical, and *nitH*, flavin oxidoreductase. A σ-54-type transcriptional regulator (*nitA*) predicted to respond to nitrogen limitation is typically found nearby [15]. Despite having been described some 15 years ago [16], the biological function of the Nit1C gene cluster in bacteria has remained obscure. However, recently a genetic linkage between the cluster and the unusual ability of bacteria known as cyanotrophs to grow on toxic cyanide as the sole nitrogen source was reported [15,17]. Although Nit1C is found in a relatively small number of bacterial genomes, it is widely distributed across phylogenetic lines being present in Gram negative, Gram positive, and cyanobacteria [15]. The genes notably, are absent from members of the Archaea. Aside from its linkage to bacterial growth on cyanide as the sole nitrogen source a connection between the *nitB* gene and the cellular redox state in the cyanobacterium, *Synechocystis* sp. PCC6803, has also been reported [18,19].

In the course of studies on the cyanotroph, *Pseudomonas fluorescens* NCIMB 11764, we observed that a mutant strain in which the *nitC* gene was deleted could no longer grow on cyanide [15]. Similar observations were made with another cyanotroph (*P. pseudoalcaligenes* CECT5344) from another laboratory [17] thus, pointing to the essentiality of the canonical nitrilase for growth on cyanide. To learn more about NitC and the molecular basis of its essentiality for cyanotrophy, we cloned the *nitC* gene from strain Pf11764 and expressed the enzyme in *E. coli.* Here we describe the purification and characterization of the his-tagged Pf11764 enzyme (hereafter referred to as Nit11764) and compare its properties with those of Nit6803, the current reference for the homologous family of proteins, *nitrile_sll0784*.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma Chemical Co unless otherwise indicated. *trans*-Crotononitrile (20%, *cis*), 3-ethoxyacrylonitrile (*trans*, *cis* mixture), 2-cyanopyrmidine, 2-pentenenitrile and acrylonitrile were purchased from Tokyo Chemical Industies (TCI). Glutaronitrile was purchased from Alfa-Aesar and benzonitrile was from Across Chemicals.

2.2. Cloning and expression of nitC

The *nitC* gene (locus B723_21865) (WP_017338926.1) from Pf11764 was amplified from Pf11764 genomic DNA using 5′-GGATCCATGCCCAATGTGGACATTGCCCA-3′ and 5′-CGCACCGCTCCGAACGCTTGTACTTATCTTCGAA-3′ as forward and reverse primers, respectively. The amplified gene product was cloned into the TOPO plasmid vector, pET101 (Invitrogen) by directional cloning creating plasmid recombinant pET101:*nitC* containing a his-tag at the C-terminus (designated pGK2, see Fig. S1). For protein expression, a single colony grown on Lennox agar plates (Bacto tryptone:yeast extract:NaCl (10:5:5 g L^{-1}) [20] containing 50 μg ml^{-1} (Ap^{50}) ampicillin was placed into 5 ml of the same liquid medium containing Ap^{50} and the culture incubated for 3–4 h at which time 5 μl was placed into a new tube of the same medium and the newly inoculated culture allowed to incubate at 37 °C overnight. Five ml of the overnight culture was transferred to 1 L of LB containing Ap^{50} and 10 mM glucose and cells cultivated at 37 °C on a gyrotory shaker until the OD reached 0.5. At this time the culture was chilled on ice for 20 min after which IPTG (0.2–1 mM) was added and the culture incubated overnight before cells were harvested and stored at −80 °C. Frozen cells were suspended in lysis buffer (Buffer A) (20 mM HEPES, pH 7.5, 20 mM imidazole, 500 mM NaCl and 10% glycerol) and broken in a French Press at 20,000 psi. The cell lysate was incubated with DNase (50 μg ml^{-1}) for 10 min at room temperature before being centrifuged at 28,000×*g* for 28 min at 4 °C. The resultant supernatant was then further centrifuged at150,000×*g* after which the supernatant was collected and passed through a 0.45 μm filter before being applied to a Hi-Trap nickel-affinity column (GE Health Sciences) attached to an ÄKTA (Amersham-Pharmacia) Protein Puification System. The column was equilibrated first with Buffer A before eluting proteins with Buffer B (20 mM HEPES (pH 7.5), 200 mM imidazole, 0.5 M NaCl and 10% glycerol). Fractions containing Nit11764 were identified by detecting the formation of ammonia after having been incubated with fumaronitrile for 20–30 min as further described below. Fractions exhibiting activity were pooled, exchanged with Buffer C ($Hepes^{20}$, pH 8.0, 150 mM NaCl, 10% glycerol), and concentrated by centrifugation in a 10 K MWCO ultrafiltration membrane ($MilliporeSigma^{R}$) before being loaded on a Superdex 200 gel-filtration column (GE Healthsciences). Proteins were applied to the column and eluted at a flow rate of 0.5 ml min^{-1} with Buffer C before being frozen at −80 °C. The protein content of collected fractions was determined by the modified Lowry method [21].

2.3. Nit11764 enzyme assay

Both end-point and time-dependent assays were performed to determine Nit11764 enzyme activity. For the former, reactions were conducted in 50 mM Na_2HPO_4–KH_2PO_4 phosphate buffer (pH 7.0) (Na–K buffer) with samples being drawn at desired intervals and analyzed for the accumulation of ammonia. Ammonia was determined colorimetrically by the Berthelot procedure [22] as previously described [23]. Time-dependent assays were conducted spectrophotometrically as described for other ammonia-generating enzymes [24] by coupling the activity of Nit11764 to NADH-dependent glutamate dehydrogenase. Reaction mixtures (0.5 ml) contained 40 mM Na–K phosphate buffer (pH 7.0), 7 mM α-ketoglutarate, 0.2 mM NADH, 10 mM nitrile, 14 U, glutamate dehydrogenase, and enzyme (3–20 μg ml^{-1}). Reactions were initiated with the purified enzyme and the rate of NADH disappearance quantified using an extinction value of 6.22 mM^{-1} at 340 nm. The dinitrile, fumaronitrile, served as the substrate for routine measurement of Nit11764 activity in cell extracts.

Initial substrate screens of Nit11764 were performed by incubating the purified enzyme and determining the amount of ammonia accumulated after 1 h incubation at 30 °C. Reaction mixtures (50 μl) contained

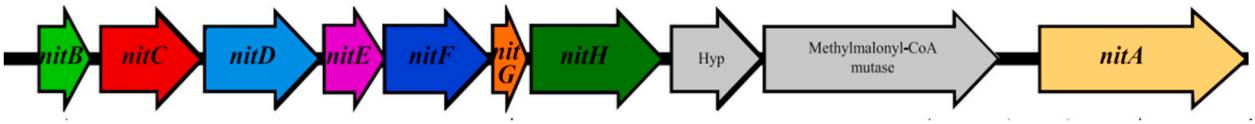

Fig. 1. Structural organization of the Nit1C gene cluster in *P. fluorescens* NCIMB 11764. Annotations are as follows: *nitA*, sigma (σ54) transcriptional activator; *nitB*, hypothetical; *nitC*, nitrilase; *nitD*, radical S-adenosylmethionine (SAM); *nitE*, Gcn5-related (GNAT) *N*-acetyltransferase; *nitF*, 5-aminoimidizole ribonucleotide synthase; *nitG*, hypothetical; *nitH*, predicted flavoprotein monooxygenase.

10 mM nitrile, 45 mM Na–K phosphate buffer (pH 7.0), and purified enzyme (20 μg ml^{-1}). Nitrile stock solutions were prepared either in Na–K buffer (pH 7.0) or the same buffer containing 10% methanol. Water insoluble substrates such as cinnamonitrile were dissolved in *N*,*N*′-dimethylformamide (DMF) with the final DMF concentration in reaction mixtures being ≤ 1%.

2.4. Sequence comparisons, homology modeling and docking studies

A sequence similarity network of Branch 1 nitrilases was generated using Cytoscape (http://www.cytoscape.org), UniProt Version: 2020_02, InterPro Version: 79 (run date 06/13/2020)) [25]. Multiple sequence alignment of Nit6803 homologs was performed using Clustal W (https://www.ebi.ac.uk/Tools/msa/clustalo/). The comparative modeling for Nit11764 was carried out with the SWISS-MODEL server [26] using the structure of Nit6803 nitrilase (PDB ID: 3WUY) as a template. The three dimensional structural model of Nit11764 was generated based on optimal sequence alignment and structural comparison with Nit6803. The molecular docking of the substrate cinnamonitrile to Nit11764 was carried out using the automated docking program, AUTODOCK [27]. Only minor manual adjustments of the modeling solution were made before the model was analyzed using graphics program COOT [28]. Figures were prepared with PyMOL (The PyMOL Molecular Graphics System, Schrödinger, LLC).

2.5. In situ NMR experiments

All ^{1}H NMR spectra were recorded on a Agilent VNMRSYS 400 narrow bore superconducting magnet spectrometer with proton resonance frequency at 399.77 MHz and a 5-mm high resolution probe. For a lock a D_2O vortex capillary was added to the NMR tube to avoid 1H/D exchange reactions. The huge water signal was suppressed using the presaturation methods. During measurements the tube was rotated at 20 Hz/s. The acquisition parameters were as follows: 45° proton pulse, relaxation delay 2 s, acquisition time 2 s and 8 repetitions, 120 s time interval for the arrayed acquisitions made over time.

3. Results

3.1. Bioinformatic analysis and modeled structure of Nit11764

A sequence similarity network constructed for proteins in Branch 1 of the nitrilase superfamily (Evalue cutoff 10^{-70} of 11,215 non-redundant protein sequences (Fig. 2)) shows that Nit11764 and Nit6803 belong to a cluster of proteins (*nitrile_s110784 family, Accession,* TIGR04048) (www.ncbi.nlm.nih.gov) comprised of 496 nodes (Fig. 2, highlighted in yellow). The genes encoding enzymes in the family reside almost exclusively in the conserved Nit1C gene cluster. The inset to Fig. 2 shows the highly conserved (100%) signature amino acid sequence surrounding the putative active site cysteine for several enzymes from different cyanotrophs (complete alignments are shown in Fig. S2). The three-dimensional structure of Nit11764 was modeled against Nit6803 (PDBID: 3WUY) which shares 64% amino acid sequence identity with Nit11764. The modeled three-dimensional structure of Nit11764 protein is very similar to that of Nit6803 with an αββα-fold and a conserved catalytic tetrad Glu44, Lys126, Cys160, and Glu133 (see Fig. 5).

3.2. Protein expression and purification

Expression of Nit11764 was toxic to *E. coli* BL21 DE3 as evidenced by a slower growth rate following IPTG induction for cells containing pGK2 versus those without (Fig. 3). Moreover, the slower growth rate coincided with the time-course of Nit11764 expression following IPTG induction and with the appearance of inclusion bodies as revealed by microscopic examination (Fig. 3 inset). While the latter are typically associated with misfolded proteins devoid of activity, specific activities

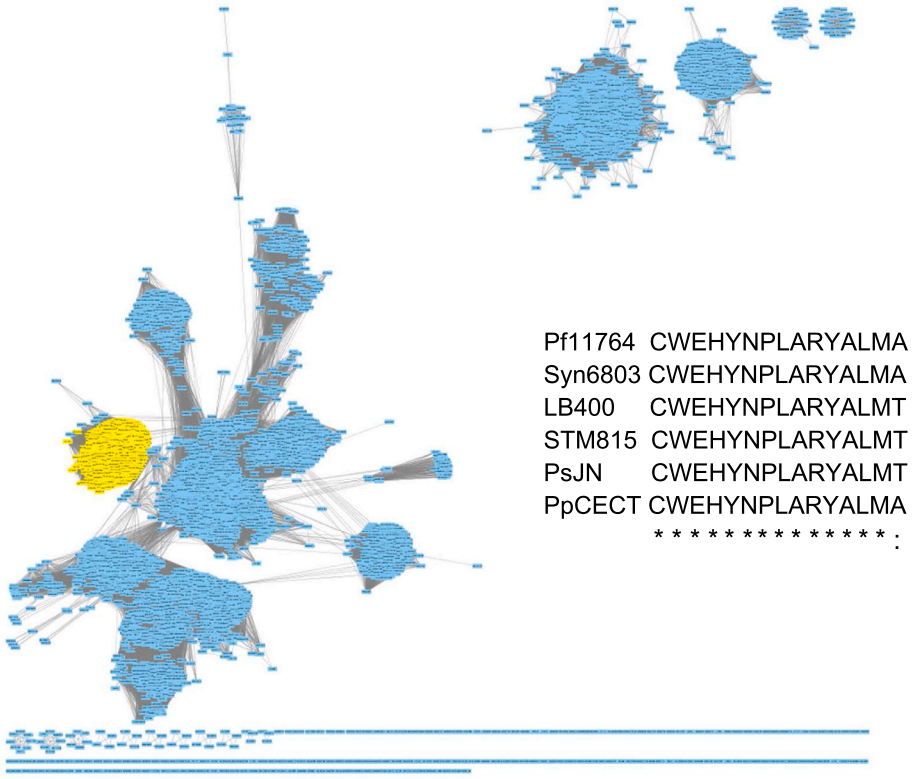

Fig. 2. Sequence network map of proteins in Branch 1 of the nitrilase superfamily at an *E* value cutoff of 10^{-70}. Metabolic networking predicts the Nit1C cluster-derived nitrilase (NitC in yellow) to be functionally exclusive. Each node (colored square) in the network contains sequences that share ≥ 90% amino acid sequence identity over any length of the sequence. The insert shows conserved sequences adjacent to the conserved cysteine for several NitC enzymes. Strain designations are as follows: Pf11764, *P. fluorescens* NCIMB 11764; Syn6803, *Synechocystis* sp. PCC6803 (source of Nit6803); LB400, *Burkholderia xenovorans* LB400, STM815, *B. phymatum* STM815; PsJN, *B. phytofirmans* PsJn; PpCECT, *P. pseudoalcaligenes* CECT5344. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

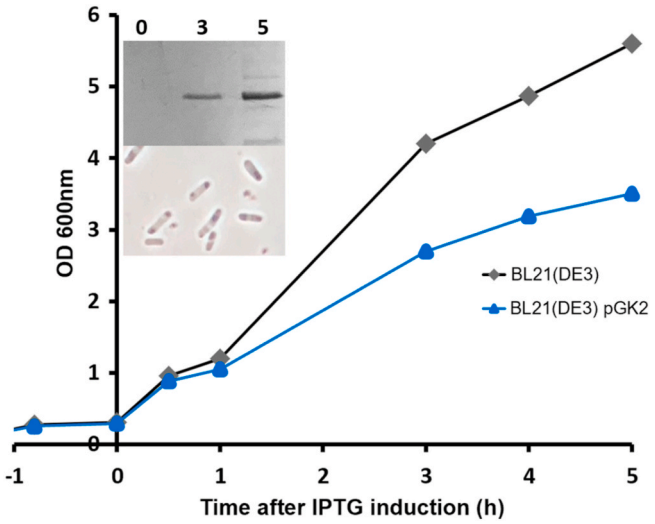

Fig. 3. Nit11764 is toxic to *E. coli*. *E. coli* BL21 DE3 cells either without or with the Nit11764-expressing pGK2 plasmid were cultivated in LB at 37 °C with shaking a 250 rpm, and at the times indicated the OD_{600nm} was read and cells fractionated by standard procedures (Invitrogen^{R}). The top inset shows SDS-PAGE of the pellet fractions collected from fractionated cells at the times indicated. The photomicrograph was taken after 5 h of induction showing extensive inclusion bodies.

ranging from 2-10 U mg^{-1} protein were typically recovered in cell extracts when assayed with fumaronitrile as the substrate. Interestingly, the majority of the activity was located in the pellet fraction when cell extracts were centrifuged at high-speed (150,000×*g*) which is consistent with the expected enrichment of inclusion bodies in the pellet. Because, Nit11764 and its nitrilase counterparts are not known to associate with the cell membrane (also expected to be recovered in 150,000×*g* pellets), we interpret these results as suggesting that the inclusion bodies formed contain active protein. While unusual, the isolation of active proteins from inclusion bodies is not unprecedented [29,30], however, to our knowledge it has not been reported for nitrilase enzymes. Nit11764 was purified by a combination of nickel-affinity and size-exclusion chromatography. In the latter case, most of the activity eluted in the void volume which is consistent with the protein assuming a large oligomeric structure as reported for other nitrilases [10,11]. Fractions exhibiting activity after size-exclusion chromatography contained a prominent band on SDS-gels with a mass consistent with that of the translated gene (34,543.47 Da plus 2262.52 derived from the fused V5 epitope plus 6-histidine tag (Fig. S1).

3.3. Substrate selectivity of Nit11764

Substrates capable of being recognized by the enzyme were initially screened by determining the extent of conversion to ammonia after 1 h incubation. The dinitrile, fumaronitrile, and the aromatic nitrile, cinnamonitrile were the best substrates identified. Other compound acted upon to variable extents included, 3- and 4-cyanopyridine, succinonitrile, sebaconitrile (octanoic acid dinitirle), *trans* -crotononitrile, *trans*-3-ethoxyacrylonitrile, *trans*-3,4-dimethoxycinnamonitrile, and 2-cyanopyrimidine (Fig. 4). Cyanide was a poor substrate. Compounds for which no bioconversion was detected included, acetonitrile, acrylonitrile, valeronitrile, β-cyanoalanine, acetone cyanohydrin, mandelonitrile, benzonitrile, 2-cyanophenol, indoleacetonitrile, 5-aminoimidazole carbo-nitrile, *tr*ans-3-hexenenitrile, 2-pentenenitrile, and glutaronitrile.

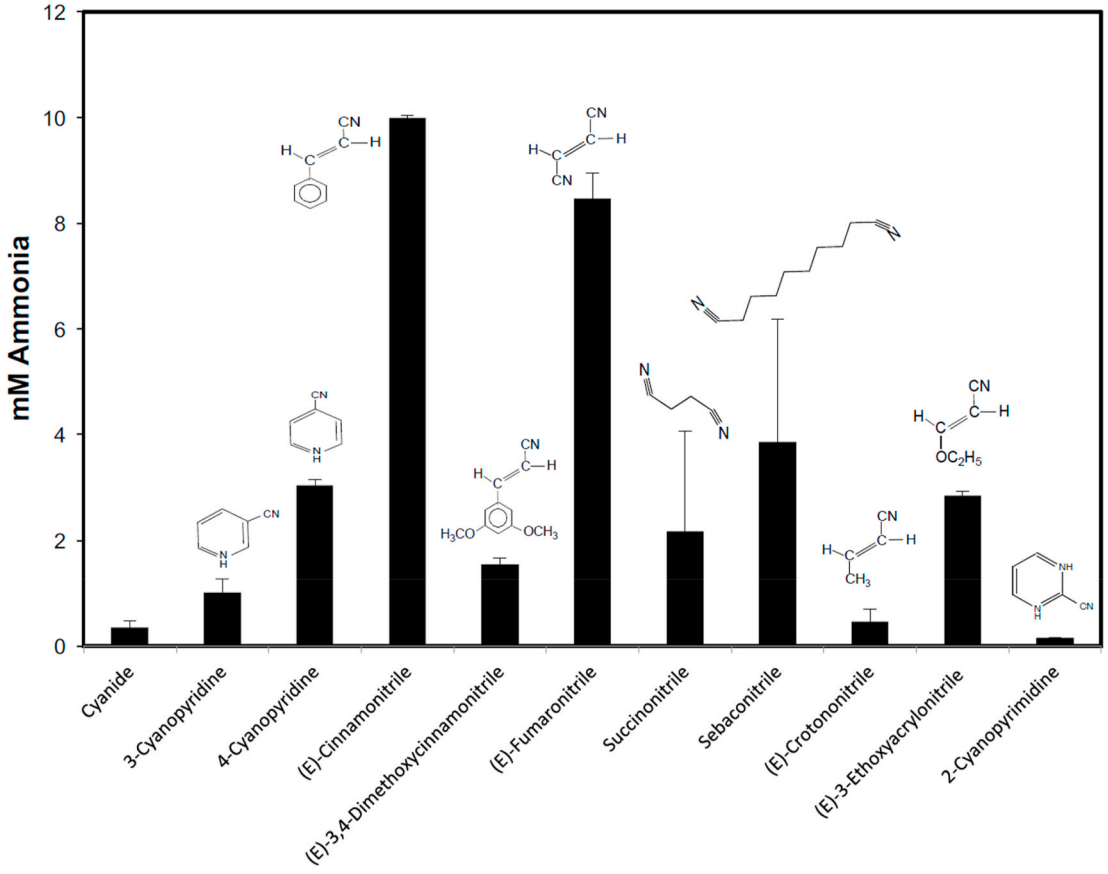

Fig. 4. Enzymatic conversion of various nitriles by purified Nit11764. Reactions were incubated at 30 °C for 1 h after which time the amount of ammonia formed was determined. Reaction mixtures (50 μl) contained 50 mM Na–K phosphate buffer (pH 7.0), 20 μg ml^{-1} protein and 10 mM substrate. The results shown represent the mean and standard deviations from the mean for three separate experiments.

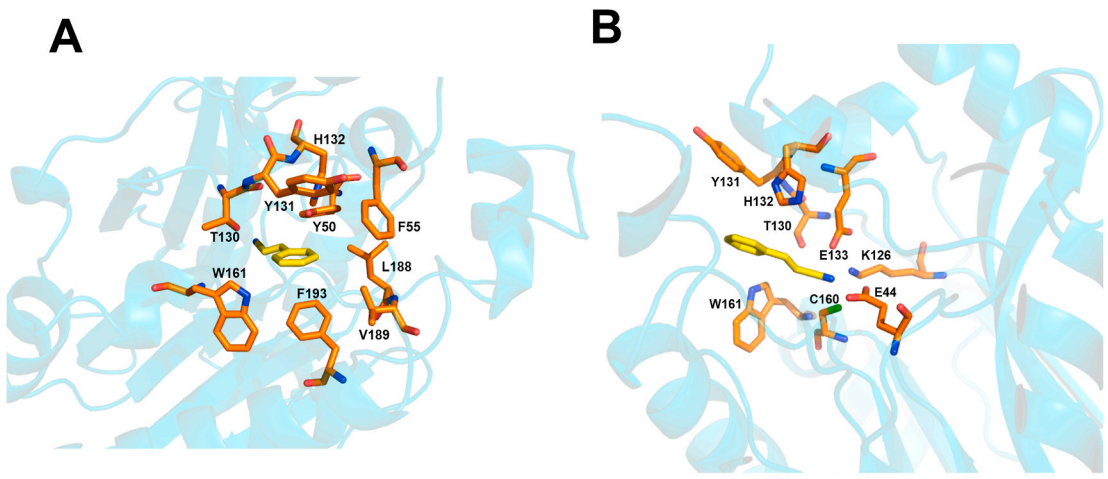

Fig. 5. Molecular docking of the Nit11764 nitrilase. (A) Cinnamonitrile docked to Nit11764. (B) Same as A showing the substrate binding pocket with adjacent residues forming the catalytic tetrad (E44, K126, C160 and E133) in stick models. The substrate binding pocket is lined with hydrophobic residues. The substrate cinnamonitrile is shown as a yellow stick model and key residues as orange stick models. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Notably, fumaronitirle was also reported to be the best substrate in two separate studies previously performed with Nit6803 [14,31]. Table 1 shows kinetic constants determined for Nit11764 compared with values previously reported for Nit6803. The affinity for fumaronitrile (Km = ~9 mM) by Nit11764 is consistent with that reported for Nit6803, but values obtained for the V_{max}, k_{cat} and catalytic efficiency (k_{cat}/K_m) of Nit11764 were collectively higher. We hypothesize this has more to do with how enzyme activity was measured as opposed to real differences between the two enzymes. For example, activity assays as previously reported for Nit6803 [14,31] were carried out by quantifying the reaction prodfuct ammonia colorimetricslly. This requires the physical removal of samples from reaction mixtures at defined set points. In contrast, we adapted an assay method used widely for assaying other ammonia-forming enzymes [24] by coupling nitrilase activity to glutamate dehydrogenase. This allows the rate of ammonia formation to be determined in real time providing a better means for determining kinetic constants. Notably, use of the spectrophotometric method for measuring the activity for some of the poorer substrates was precluded by the slowness with which ammonia accumulated. However, it is interesting that for one of the more poorer substrates, namely, 3-cyanopyridine, an estimate of the activity (0.85 U mg^{-1}) based on end-point colorimetric determination of ammonia gave a similar value as that reported for Nit6803 [14]. We similarly determined the kinertic constants for Nit11764 with cinnamonitrile, which from our initial screen and determination of the K_m (1.27 mM ± 0.4 mM) suggested that it was an even better substrate than fumaronitrile. However, because of the low turnover rate (k_{cat}) the catalytic efficiency of Nit11764 towards cinnamonitrile is not that much different from that observed for fumaronitrile. A comparison between Nit11764 and Nit6803 when it comes to cinnamonitrile could not be made since it was not previously reported as having been tested as a substrate for Nit6803 [14,31]. Fig. 4 shows that in contrast to cinnamonitrile, 3,4-dimethoxycinnamonitrile underwent a significantly lower conversion to ammonia, the bonded methoxyl substituents possibly hindering access to the binding pocket.

Table 1
Comparison of kinetic constants for nitriles by Nit11764 and Nit6803 enzymes.

Enzyme	Substrate	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
Nit11764	Fumaronitrile	72.6 ± 8.5	45.22 ± 2.8 s^{-1}	8.57 ± 0.8	(5.36 ± 0.9) X 10^3
	Cinnamonitrile	14.6 ± 1.6	9.79 ± 0.3	1.27 ± 0.4	(7.47 ± 1.8) X 10^3
	3-Cyanopyridine	0.85 ± 21	Nd	Nd	Nd
Nit6803[a]	Fumaronitrile	3.2	15.13 ± 0.16	9.01 ± 0.51	1679.25
	Cinnamonitrile	Nd	Nd	Nd	Nd
	3-Cyanopyridine	0.68	0.45 ± 0.07	3.20 ± 0.24	140.63

[a] Data from Zhang et al., [14]. Nd, Not determined.

3.4. Docking studies of Nit11764

To understand the function and substrate specificity of Nit11764, molecular docking studies were performed using the program AUTODOCK [27] (Fig. 5). The substrates cinnamonitrile and fumaronitrile each docked close to Cys160 established as the nucleophile in related nitrilases. Glu44 lies in an appropriate position to activate Cys160 by deprotonation with hydrogen bonds between Lys126, Glu44 and Cys160. A conserved Glu133 is further believed to stabilize the entire complex. The periphery of the substrate binding pocket is lined by Thr130, H132, Pro185, Leu188, V189, Tyr50, Phe55, Tyr131, Trp161, and Phe193. The hydrophobic nature of the binding pocket is consistent with that also reported for Nit6803 [14] and is believed to be an important factor in determining substrate specificity. Both cinnamonitrile and fumaronitrile fit the Nit11764 substrate binding pocket well. Furthermore, the higher affinity of the enzyme for cinnamonitrile compared with fumaronitrile is consistent with the aromatic ring of cinnamonitrile interacting more strongly with hydrophobic residues lining the binding pocket (e.g., Tyr50, Phe55, Tyr131, Trp161, Leu188, Val189, and Phe193) (Fig. 5).

3.5. The products of fumaronitrile conversion are ammonia and the trans isomer of 3-cyanoacrylic acid

The products from the typical nitrilase-catalyzed conversion of a nitrile are ammonia and the corresponding carboxylic acid. Thus, from fumaronitrtile the dicarboxylate, fumarate, would be expected to accumulate were both of the nitrile substituents hydrolyzed. However, since a one-half equivalency between fumaronitrile supplied and ammonia formed was observed (Fig. 4) the monocarboxylate would be expected to accumulate. To identify the carboxylate, Nit11764 was incubated with fumarontrile and the course of the reaction followed by ^{1}HNMR. As illustrated in Fig. 6, the equivalent protons in fumaronitrile (6.48 ppm)

decreased in intensity accompanied by a concomitant increase in the appearance of a pair of doublets (6.71, 6.66 and 6.04, 5.99 ppm) each with a coupling constant (*J*) of 16.6 Hz (1H, in HO_2C–CH=CH–CN and 1H, in HO_2C–CH=CHCN). These correspond to the monocarboxylate, *trans*-3-cyanopropenoic (3-cyanoacrylic acid). The *trans* configuration was implied from a comparison of the spectrum with commercial ethyl-β-*cis*-3-cyano-2-propenoic acid. In this case a similar pair of doublets was observed (6.64, 6.61 and 6.09, 6.06) corresponding respectively, to 1H in $H_3CCH_2O_2C$–CH=CH–CN and 1H in $H_3CCH_2O_2C$–CH=CH–CN but having a coupling constant of only 11.4 Hz indicative of the *cis*-configuration (data not shown). Accordingly, the higher value of 16.6 Hz for the bioconversion product gave strong reason to conclude that the *trans* not *cis* isomer was represented. A second biological product was also detected (6.89, 6.85 and 6.31, 6.27 ppm, Jvalue 16.0 Hz) albeit in trace quantity. We hypothesized that this was likely the precursor of 3-cyanoacrylate, namely 3-cyanoacrylamide (Fig. 6). This hypothesis was reinforced by comparing the ^{1}HNMR spectrum of the biologically-formed product with that of commercial *trans*-3-carboxyacrylamide (fumaric acid monoamide) and its *cis* counterpart (maleic acid monamide). Each commercial compound yielded a similar pair of doublets with chemical shift values in line with those of the biologically-formed product but with distinctly different coupling constants; 15.6 for the *trans* versus 12.4 for the *cis* species (data not shown). Accordingly, the higher value of 15.6 Hz for the bioconversion product gave reason to conclude that the *trans* isomer of the monoamide (*trans*-3-cyanoacrylamide) was formed and is consistent with the conventional mechanism of nitrilase-catalyzed reactions in which small quantities of the amide intermediate can escape from the enzyme [1,2,36].

4. Discussion

A relatively large number of nitrilases (aminohydrolases) from Branch 1 of the large nitrilase superfamily have been characterized from various biological sources. However, Nit6803 represents the singular example of a Branch 1 enzyme for which the crystal structure is known and for which the corresponding gene like that of Nit11764, resides in the conserved Nit1C gene cluster. This afforded the unique opportunity of using the Nit6803 enzyme as a model for comparison with Nit11764. As might be expected, the substrate profile and molecular structure of the two are similar. The dinitrile, fumaronitrile, the best substrate previously identified for Nit6803 [14,31], ranked second only to the aromatic nitrile cinnamonitrile for Nit11764. Benzonitrile was not a substrate for Nit11764 in contrast to results reported for Nit6803 [14, 31]. The affinity for cinnamonitrile by Nit11764 (K_m = 1.27 mM) was almost 7 times greater than that of fumaronitrile presumably because it interacts more favorably with hydrophobic residues lining the binding pocket. The same rationale likely accounts for the strong activity towards the more hydrophobic C8 dinitrile, sebaconitrile (Fig. 4). Top and side views of the binding pocket docked with cinnamonitrile show a good fit (Fig. 5) with the cyano group oriented in close proximity to the active site cysteine (Cys160). Other members of the catalytic triad (E44, K126, [E133]) are positioned nearby. We hypothesize that the reduced activity towards 3,4-dimethoxycinnamonitrile (Fig. 4) has to do with its size; the methoxyl groups bonded to the aromatic ring possibly hindering entry into the binding pocket. It is interesting that the majority of the compounds recognized by the enzyme have a flat to planar conformation: e.g., fumaronitrile, cinnamonitrile, crotononitrile, 3-ethoxynitrile, 2-cyano- and 4-cyanopyridine. Furthermore, the cyano group for each of these compounds occupies a vinylic position (alpha to the double bond). The significance of these structural characteristics is unknown at present but could be important in future studies aimed at further defining the basis of Nitt11764's substrate selectivity and that of its homologs.

Recent studies have shown that a significant factor contributing to

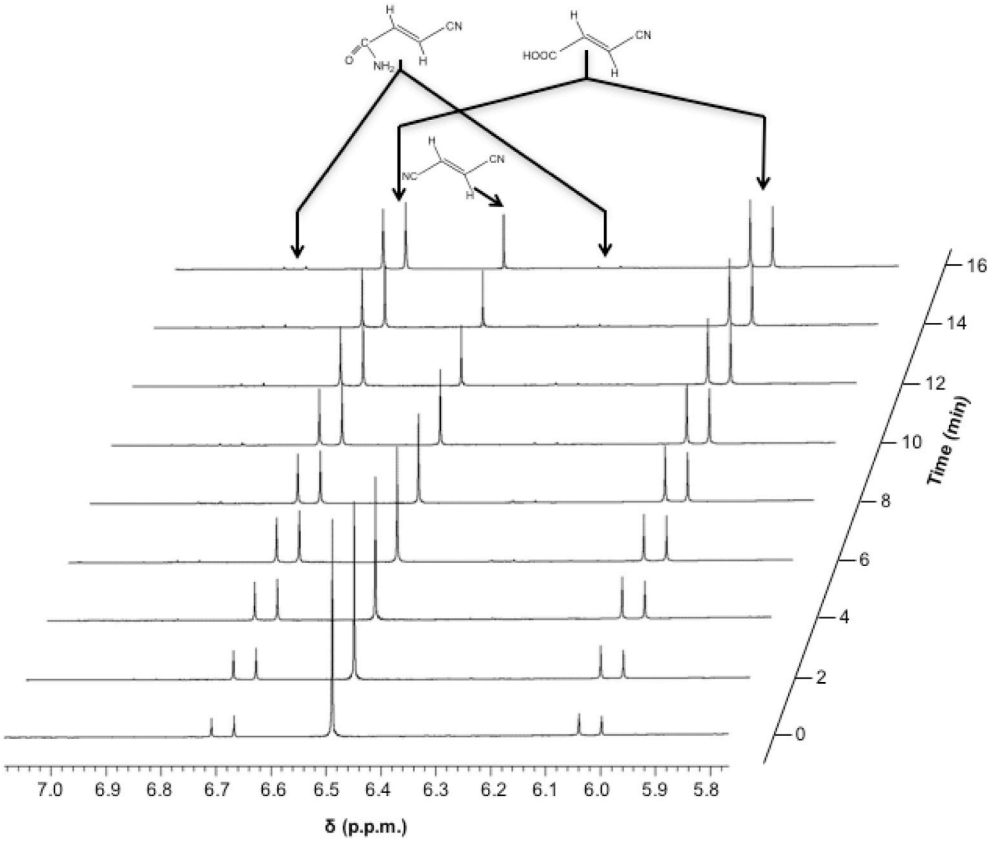

Fig. 6. ***In situ*** **^{1}HNMR analysis of fumaronitrile bioconversion by Nit11764.** Reaction mixtures were 20% deuterium enriched in H_2O and contained 30 mM fumaronitrile, 0.1 mg ml^{-1} NitC11764 and 5 mM Na–K phosphate buffer (pH 7.0).

the substrate specificity of Branch1 nitrilases is their quaternary structure consisting of oligomeric left-handed helical spirals or twisted filaments comprised of interacting dimers [11–13]. Nit6803, for example, has been described as a spiral with a small twist (relaxed) ($\Delta_\Psi \sim -60°$) which has been equated with broad specificity for relatively larger substrates [12]. In contrast, other nitrilases such as the *Arabidopsis thaliana* Nit4 (AtNit4) enzyme, exhibits a large twist (tight) ($\Delta_\Psi \sim -73°$), has a narrow substrate specificity, and acts mainly on smaller substrates. AtNit4, for example, is specific for relatively small compounds such as β-cyanoalanine which is thought to be the physiological substrate [13]. We may predict that Nit11764 would also assume a small helical twist in its quaternary state because of its close homology to Nit6803. The demonstrated broad specificity for relatively large nitrile molecules is consistent with this. Moreover, like Nit6803 and other nitrilases capable of attacking dinitriles [31–36], Nit11764 also is able to act on only one of the cyano groups, the monocarboxylate being formed identified by ^{1}HNMR as *trans*-3-cyanoacrylate (Fig. 6).

5. Conclusions

While the results presented here provide experimental evidence of Nit11764 as a nitrilase, the association if any of its catalytic activity with its essentiality of Pf11764 on cyanide for growth remains elusive. Based on the relative poor activity exhibited towards cyanide it seems unlikely that its main role is one of nitrogen provision (since the conversion of cyanide would be expected to generate ammonia which bacteria can readily assimilate). In addition, the relative poor catalytic efficiencies against even the best of substrates (5-7 X 10 $M^{-1}s^{-1}$) gives reason to question whether the nitrilase activity of Nit11764 is the sole reason for its essentiality. Indeed, as a family, the actual physiological substrates and biological roles for most nitrilase enzymes remains unknown. The ability to form large filaments could suggest a role in forming the core of a large enzyme complex the separate components of which function collectively to fulfill a common objective. Such roles have been ascribed to other filament forming enzymes [37], and given the current description of the Nit1C gene cluster as having a possible biosynthetic function (www.nim.nih.gov), the idea of a large biosynthetic metabolon with Nit11764 at the core is not inconceivable. That Nit11764 may function in a separate capacity aside from its defined enzymatic activity finds analogy with reports of other nitrilases exhibiting ancillary functions. For example, the Nit1 enzyme from *Arabadopsis thaliana*, which displays nitrilase activity towards a broad range of aliphatic and aromatic nitriles, has been reported to participate in processes regulating cell death and development [38,39]. In a similar context, the Branch 10 Nit1 enzyme from mice and humans, first recognized as having tumor suppressor activity [4,40] is known to suppress T-cell proliferation [41] and to interact with proteins involved in the cateninWnt/β-catenin signaling pathway controlling tumorigenesis and neurodegenerative disease [42]. Thus, it is not inconceivable, given the toxicity of cyanide that Nit11764 confers some function separate from its ability to catalyze the hydrolysis of nitriles that is essential to cyanide utilization as a nutritional nitrogen source. Future studies will probe this question and are expected to extend our knowledge of this rather diverse group of enzymes found throughout bacteria, plant, and animal taxa.

CRediT authorship contribution statement

Lauren B. Jones: Conceptualization, Data curation, Formal analysis, Writing - review & editing. **Xiaoqiang Wang:** Methodology, Formal analysis, Writing - review & editing. **Jaya S. Gullapalli:** Data curation, Visualization. **Daniel A. Kunz:** Conceptualization, Data curation, Formal analysis, Writing - review & editing.

Declaration of competing interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

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