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A novel thermostable nitrile hydratase

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Abstract A novel, nitrile-degrading, thermophilic microorganism belonging to the genus *Bacillus* and most closely related to strain DSM 2349 has been isolated. The strain grew optimally at 65°C with the constitutive expression of a thermostable intracellular nitrile hydratase. No aromatic-specific "benzonitrilase" activity was detected under any conditions. The enzyme, an $\alpha_2\beta_2$ heterotetramer with a native molecular weight of 110 kDa, was purified to homogeneity. N-terminal sequence data showed no homology to known bacterial α subunit sequences but had a high level of identity with other bacterial N-terminal β subunit sequences. The purified enzyme had a broad pH-activity range (50% activity limits were pH 5.1 and 8.7) and was stable in aqueous solution up to 60°C in the absence of either substrates or substrate analogues. Substrate specificity was restricted to aliphatic nitriles, but an unusual preference for branched and cyclic aliphatic nitriles was noted. Turnover rates under optimum reaction conditions were 746 and 4580 sec^{-1} for acetonitrile and valeronitrile, respectively.

Key words Thermophile · Bacillus · Nitrile hydratase · Thermostable

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Introduction

Nitrile hydratases (EC 4.2.1.84) catalyze the hydration of nitriles to the corresponding amides. This activity has been isolated from a number of different bacterial sources: *Arthrobacter* (Ramakrishna and Desai 1992), *Bacillus* (Cramp et al. 1997), *Comamonas* (Cerbelaud et al. 1995), *Corynebacterium* (Amarant et al. 1989; Tani et al. 1989), *Rhodococcus* (Endo and Watanabe 1989; Kobayashi et al. 1992; Nagasawa et al. 1988), *Pseudonocardia* (Yamaki et al. 1997), and *Pseudomonas* (Nagasawa et al. 1987; Nawaz and Chapatwala 1991). However, it is not ubiquitously distributed. The enzymes are typically composed of two different subunits (α and β) which form αβ or $\alpha_2\beta_2$ functional proteins, the catalytic center lying at the α–β interface. Two different catalytic center configurations are known: nonheme Fe^{III}-S and noncorrinoid Co complexes. A subset of the Fe^{III}-S-containing enzymes are activated by light in a photoinduced denitrosylation (Bonnet et al. 1997; Odaka et al. 1997).

Nitrile hydratases are important industrial enzymes, being used in the 30-kton/year production of acrylamide (Yamada and Kobayashi 1996) and in the treatment of organocyanide industrial effluents (Wyatt and Knowles 1996). The future application of nitrile hydratases in the fine chemicals (Bauer et al. 1994; Eyal and Charles 1990; Finnegan et al. 1992; Gilligan et al. 1993; Hashimoto et al. 1996; Maddrell et al. 1996; Mauger et al. 1988; Meth-Cohn and Wang 1997; Warhurst and Fewson 1994) and pharmaceuticals (Layh et al. 1994; Prepechalova et al. 1996) industries is also of rapidly growing interest.

Most mesophilic nitrile hydratases are relatively unstable in the absence of substrates or substrate analogues. The possibility of employing more stable enzymes at higher temperatures or in the presence of denaturants such as organic solvents has led to a search for thermophilic homologues (Cramp et al. 1997; Takashima 1995; Yamaki et al. 1997). Here, we report the isolation and partial characterization of a novel, thermostable nitrile hydratase derived from a moderately thermophilic bacterium.

Materials and methods

Materials

Liquid chromatography matrices and equipment for protein purification were obtained from Pharmacia (Uppsala, Sweden). All other chemicals used were from commercial sources and of analytical grade.

Media

The defined basal media used for the isolation of the acetonitrile-utilizing micro-organisms contained the following components: KH_2PO_4, 2 g/l; NaCl, 1 g/l; $MgSO_4.7H_20$, 10 mg/l; $(NH_4)_2SO_4$, 8.6 g/l; $FeSO_4.7H_2O$, 10 mg/l; thiamine, 0.4 mg/l; biotin, 2 μg/l; inositol, 2 mg/l; and sodium succinate, 20 mM. The pH of the basal medium was adjusted to pH 7.5 with NaOH before autoclaving. Solid basal medium contained 1.5% Bacto agar (Difco, Detroit, MI, USA). Where nitriles were used as the sole nitrogen and/or carbon source, succinate and $(NH_4)_2SO_4$ were omitted, as appropriate. Nitrile substrates were used at a concentration of 25 mM. Nutrient Broth No. 2 (Oxoid), was used for routine growth of isolates and for the production of cell biomass.

Enrichment and isolation of bacteria

Soil samples were stored at 4°C after collection. Enrichments were performed by adding 1 ml of an aqueous soil suspension to 50 ml of basal media containing acetonitrile as a sole C and/or N source. Inoculated media were incubated in 250-ml Erlenmeyer flasks at 60°C with orbital shaking (300 rpm). At 24-h intervals, the enrichments were sampled and aliquots spread on selective solid basal media, incubated at the same temperature. Single colonies were selected and restreaked until pure cultures were obtained. Pure cultures were grown in 50-ml volumes of basal medium with and without nitrile additions. Growth of bacterial cultures was monitored spectrophotometrically at 600 nm with a Cecil spectrophotometer.

Identification and microscopy

Gram staining was carried out according to Norris and Swain (1971). For electron microscopy, a cell suspension was prepared from a plate culture by gentle shaking with a 0.9% (w/v) NaCl solution. Cells were then centrifuged, washed, resuspended in distilled water, transferred to carbon-coated grids, and negatively stained with 2% (w/v) uranyl acetate (pH 7.2) containing 0.1% (w/v) bovine serum albumin. Electron micrographs were taken with a Jeol 10/10 scanning electron microscope, operating at an instrument magnification of 12 000.

16S rDNA sequence determination and analysis

Genomic DNA extraction, polymerase chain reaction (PCR) amplification of 16S rDNA, and 16S rDNA sequence determination were carried out using procedures described by Rainey et al. (1996). The 16S rDNA sequence was manually aligned against representatives of the thermophilic members of the genus *Bacillus* using the ae2 editor (Maidak et al. 1994). Pairwise similarity values were calculated within the ae2 editor (Maidak et al. 1994).

Nucleotide sequence accession numbers

The 16S rDNA sequence determined in this study has been deposited in the EBI database under the accession number Y15569. The accession numbers of the reference strains used in the sequence comparison are as follows: *Bacillus* sp. (starch-negative) (DSM 2349), Z26929; *Bacillus pallidus* (DSM 3670), Z26930; *"Bacillus thermoalkalophilus"* (DSM 6866), Z26931.

Nitrilase assay

The release of ammonia from the conversion of nitriles to the corresponding acids was assayed using the phenol-hypochlorite ammonia detection method (Fawcett and Scott 1960). The reaction mixture (300 μl) contained 25 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.2), 25 mM acetonitrile, and a specified volume of enzyme solution or whole cell suspension. All assays were carried out at 60°C. Aliquots (100-μl) of the reaction mixture were removed at intervals up to 10 min and immediately quenched by addition of 400 μl of solution A (0.59 M phenol and 1 mM sodium nitroprusside). After mixing, 400 μl of solution B (0.11 M sodium hypochlorite and 2 M NaOH) was added and the mixture allowed to stand for 30 min at room temperature. The absorbance was measured at 600 nm.

When assaying pure or partially purified nitrile hydratase, the reaction mixture was supplemented with an excess of *Bacillus* strain RAPc8 amidase partially purified by Q-Sepharose and phenyl-Sepharose chromatography steps (data not shown).

One unit of enzyme activity was defined as the amount of enzyme which catalyzed the release of 1 μmol of NH_3 per min under standard assay conditions.

Protein determination

Protein concentration was determined using the Bradford Coomassie Brilliant Blue dye binding assay (Bradford 1976) (Bio-Rad Laboratories, Hercules, CA, USA). Protein standards were prepared using bovine serum albumin (Fraction V, Sigma, St. Louis, MO, USA).

Fermentation

Cell biomass was generated by batch fermentation in a 10-l fermentor (LH model 210) using nutrient broth (No.2)

under the following conditions: 500 rpm stirring; 1 v/v of sterile air/min; 60°C, without pH control. Bacteria were harvested at early stationary phase by centrifugation (4000 × *g* at 4°C for 30 min). Packed cells were washed and resuspended in 25 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.2).

Purification of the *Bacillus* strain RAPc8 nitrile hydratase

All purification steps were performed at room temperature using a Pharmacia fast performance liquid chromatography (FPLC) system.

Preparation of cell free extracts

The washed cell pellet (45 g) from two 7-l fermentations was resuspended in 100 ml 50 mM KH_2PO_4/K_2HPO_4 pH 7.2 buffer and disrupted by sonication (MSE Soniprep 150). The cell debris was removed by centrifugation (25000 × *g*, 20 min) and the clarified supernatant retained for further processing.

Q-Sepharose adsorption chromatography

The clarified supernatant was loaded onto a Q-Sepharose column (2.6 cm × 12 cm) equilibrated with 50 mM KH_2PO_4/K_2HPO_4 buffer pH 7.2 at a flow rate of 2 ml/min (used for all steps). The column was washed with 3 column-volumes of the same buffer before eluting with an 8-column-volume linear salt gradient (0 M NaCl to 0.3 M NaCl in 50 mM KH_2PO_4/K_2HPO_4 pH 7.2). Active fractions were combined and diafiltered using an Amicon Ultrafiltration unit (PM30 cut-off membrane; Amicon, Lexington, MA, USA) to a final volume of 30 ml.

The concentrated active fractions from the first Q-Sepharose column were re-chromatographed under the same conditions except that the enzyme was eluted with a 7-column-volume linear gradient. Active fractions were pooled and concentrated in an Amicon Centricon bench top centrifuge concentrator (30 k cut-off) to a final volume of 2 ml.

Superdex 200 prep-grade gel permeation chromatography

A Superdex 200 column (1.6 cm × 60 cm) was equilibrated with 50 mM KH_2PO_4/K_2HPO_4 pH 7.2, 0.25 M NaCl, loaded with 0.5-ml volumes of enzyme solution, and eluted at a flow rate of 0.5 ml/min. Fractions (1 ml) were collected. Active fractions were pooled and diafiltered (as before) into 25 mM Bis-Tris buffer pH 6.9 to a final volume of 2 ml.

Chromatofocusing on Mono-P

A Pharmacia Mono P column was loaded with 1 ml 5 M NaOH and equilibrated with 25 mM Bis-Tris pH 6.9. The concentrated enzyme fractions from the previous step were loaded and washed with 3 column-volumes of the equilibration buffer. Nitrile hydratase activity was eluted with 100% Polybuffer 74 (Pharmacia ampholyte buffer) pH 3.4, which creates the linear pH gradient. The active fractions were combined and used for further characterization studies.

Analytical methods

Analytical gel electrophoresis was performed in 6% and 7.5% polyacrylamide gels with Tris-HCl buffer pH 8.8. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12.5% polyacrylamide gels. Gels were stained with Coomassie brilliant blue G-250 and destained with 12.5% isopropanol, 5% acetic acid. The relative molecular mass of the enzyme subunits was determined by comparison with the relative mobilities of the standard proteins (bovine serum albumin, 66 kDa; fumarase, 48.5 kDa; carbonic anhydrase, 29 kDa; β-lactoglobulin, 18.4 kDa; α-lactalbumin, 14.2 kDa).

Blotting and sequencing

Purified nitrile hydratase was electrophoresed on 12.5% polyacrylamide gels and transferred to Problott membranes using semi-dry blotting procedures. Membranes were stained with Coomassie Blue and protein bands sequenced using an Applied Biosystems 470A amino acid sequencer (Applied Biosystems, Foster City, CA, USA).

Substrate specificity and enzyme kinetics

Analyses of enzyme substrate specificity and reaction kinetics were carried out using gas chromatography. Enzyme reactions were carried out at 60°C in 10-ml temperature-controlled stirred glass cells (3-ml reaction volume). The reaction mixture comprised 25 mM KH_2PO_4/K_2HPO_4 buffer pH 7.2, 50 mM nitrile substrate, and an appropriate volume of purified enzyme.

Substrates and products were quantified using gas chromatography (Shimadzu GC-14b, Shimadzu, Kyoto, Japan) equipped with an RT-QPlot capillary column (30 m × 0.53 mm) which resolves nitriles, amides, and carboxylic acids. The aqueous sample (1 μl) was injected via a AOC-17 autosampler, separated with He carrier gas (oven temperature 130°–220°C), and the data stored and processed with a Shimadzu CR6A integrator.

Results

Isolation and characterization of nitrile-degrading bacteria

A thermophilic bacterial isolate capable of growth at 60°C on a variety of aliphatic nitriles as the sole nitrogen source was isolated from Australian lake sediment. A pure culture, designated strain RAPc8, was obtained by repeated cycles of plating on selective and rich media (nutrient agar) at 60°C.

On electron microscopic examination, the organism appeared as short rods of between 0.8 and 1.2 μm in width and 2–2.5 μm in length, with a pronounced capsule. Cells were sporulating, motile, and grew in nonpigmented colonies which were round and smooth with entire margins. The sporangia were slightly swollen and located in subterminal to terminal regions. Reaction to Gram staining was variable.

On both solid and liquid nutrient media, the organism did not grow at temperatures above 75°C or below 37°C (Fig. 1). The optimum growth temperature was 65°C.

Phylogenetic position

A 16S rDNA sequence comprising 1486 nucleotides between positions 45 and 1518 (*E. coli* positions) was determined for strain RAPc8. A comparison of the 16S rDNA sequence of strain RAPc8 with all available 16S rDNA sequences of the thermophilic members of the genus *Bacillus* indicated a relationship to the cluster previously shown to comprise the strains *Bacillus* sp. (starch-negative) (DSM 2349), *Bacillus pallidus* (DSM 3670), and "*Bacillus thermoalkalophilus*" (DSM 6866) (Rainey 1994). The 16S rDNA similarity values between strain RAPc8 and these strains are in the range 99.4%–99.9%. The highest 16S rDNA sequence similarity of strain RAPc8 was found to *Bacillus* sp. (starch-negative) (DSM 2349), at 99.9%. These values indicate that strain RAPc8 is a member of the thermophilic *Bacillus* cluster and most closely related to the strain DSM 2349. These relationships could be more validly established using DNA–DNA hybridization. It is not currently known whether the closely related DSM strains produce nitrile-metabolizing enzymes.

Nitrile utilization

Bacillus strain RAPc8 was unable to grow on nitriles as a sole carbon source. Growth on nitriles as a sole nitrogen source was limited to simple aliphatic compounds including acetonitrile, acrylonitrile, and, to a lesser extent, propionitrile (data not shown). Aromatic and branched aliphatic nitriles did not support growth in liquid or on solid media.

Induction

The nitrile hydratase was produced constitutively from mid-log growth phase, both in complex medium and in basal medium containing acetonitrile as a sole nitrogen source (Fig. 2). Nitrile hydratase specific activity was not enhanced on addition of various nitriles prior to inoculation or during culture growth (data not shown). The absence of growth on benzonitrile under any of the conditions tested suggests strongly that *Bacillus* strain RAPc8 does not express both aliphatic-specific nitrile hydratase and aromatic-specific benzonitrilase. Both enzimes may be expressed under appropriate conditions by many mesophilic bacteria (Amarant et al. 1989; Bianchi et al. 1993; Fallon et al. 1997).

The specific yield of nitrile hydratase/amidase activity in cell extracts was not enhanced by addition of urea (134 mM) or Co^{2+} ($0.01\,g{\cdot}l^{-1}$) to the culture medium, as has been observed for some mesophilic nitrile-degrading bacterial strains (Kobayashi et al. 1991; Nagasawa et al. 1991).

Purification

Nitrile hydratase activity was purified to homogeneity in four steps (Table 1). The eluate from Mono-P chroma-

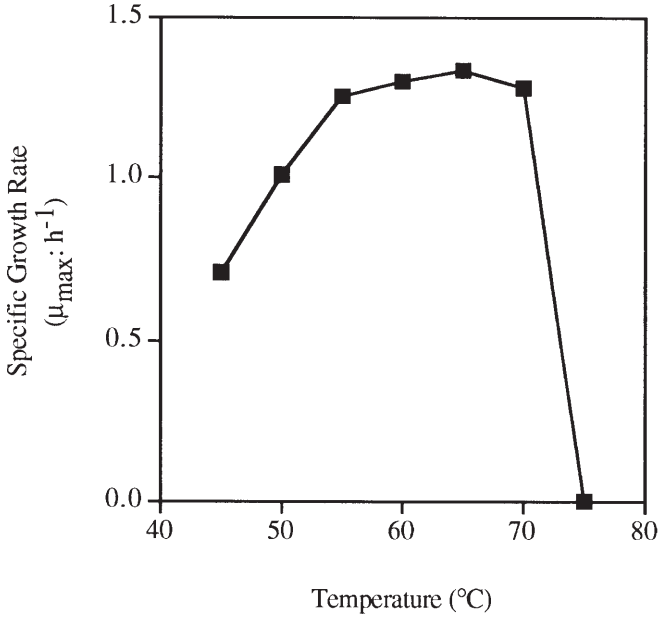


Fig. 1. Growth temperature profile μ_{max} (Maximum specific growth rate)

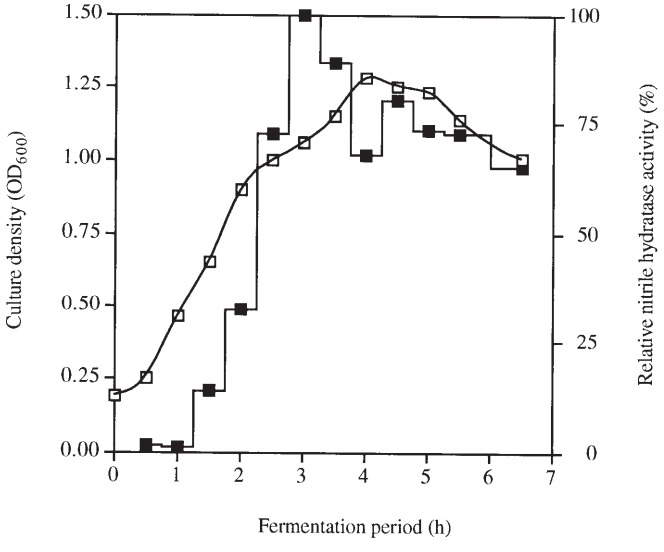


Fig. 2. Growth profile and expression of nitrile-degrading activity in *Bacillus* strain RAPc8. OD_{600} (*open squares*) and relative nitrile hydratase activity (closed squares)

tofocusing showed a single protein absorbance peak, corresponding to the activity peak (data not shown). SDS-PAGE analysis of this preparation showed two protein bands of approximately equal density, assumed to represent two distinct subunits (Fig. 3). This was confirmed by 7.5% native-PAGE where a single protein band, excised, electroeluted, and re-electrophoresed on a 12% SDS-PAGE gel, produced two distinct bands with migrations similar to a 29-kDa molecular weight marker.

The purification of the nitrile hydratase was approximately 14.6-fold, with an overall yield of approximately 3%. The specific activity of the homogeneous enzyme suggests that the nitrile hydratase was approximately 7% of the total soluble protein in *Bacillus* strain RAPc8 cells.

Molecular weight and subunit composition

The approximate relative molecular mass of the active enzyme was determined by gel filtration on a Superdex 200 column and in comparison with authentic molecular weight standards. The activity eluted routinely as a single peak

Table 1. Purification of nitrile hydratase from *Bacillus* strain RAPc8

Step[a]	Total protein (mg)	Total activity (units)	Specific activity (U/mg)	Yield %	Purification (fold)
Cell-free extract	1500	1050	0.7	100	–
Q-Sepharose 1	280	658	2.35	61	3.4
Q-Sepharose 2	60	186	3.1	17	4.4
Superdex 200	12	97.2	8.1	9	11.6
Mono P chromatofocusing	2.8	25.6	10.2	2.7	14.2

[a] Experimental details are outlined Materials and methods.

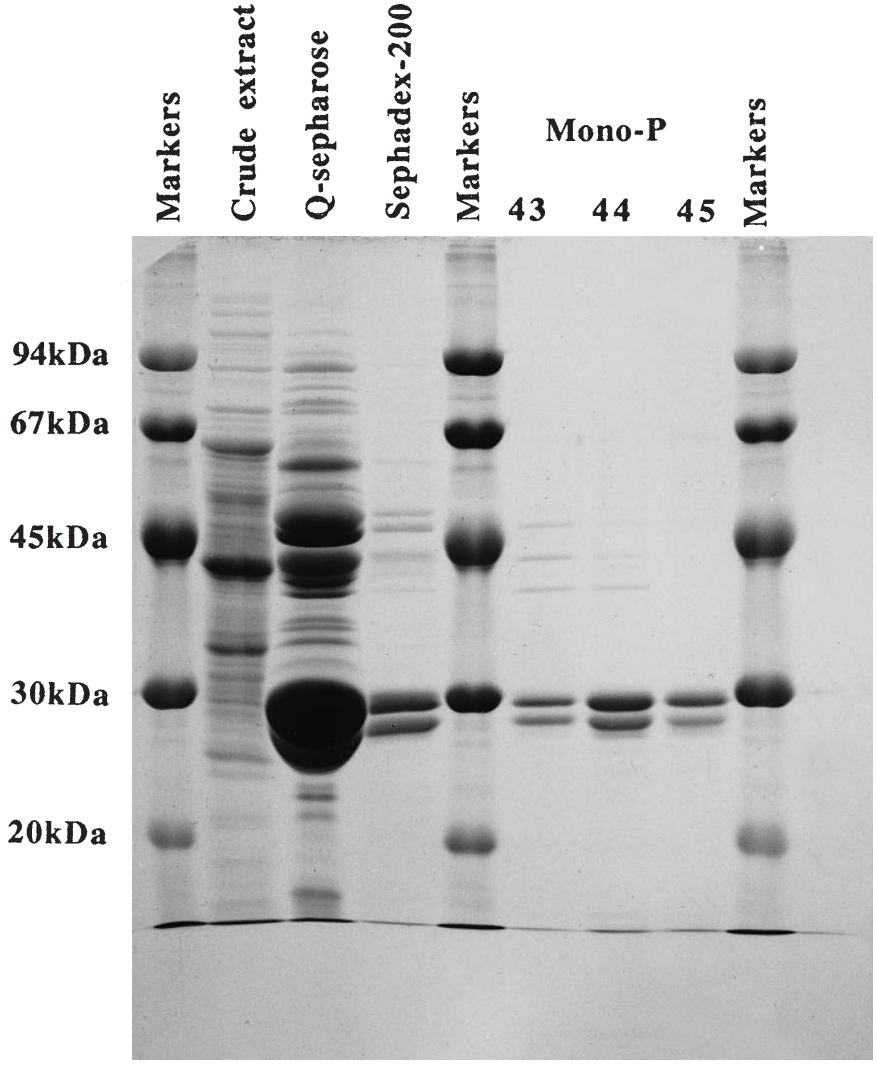


Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of purified nitrile hydratase fractions

corresponding to a relative molecular mass of approximately 110 kDa. No lower-molecular-weight activity peak was detected under any elution conditions. Migration of the homogeneous enzyme on SDS-PAGE at various acrylamide concentrations yielded bands of 28 kDa and 29 kDa (±5%), designated by convention as the α and β subunits respectively. Taken together, these data strongly suggest that the native enzyme exists as an $\alpha_2\beta_2$ tetramer. Unlike the nitrile hydratase from *Rhodococcus* sp. R312 (Huang et al. 1997), in the *Bacillus* strain RAPc8 enzyme we found no evidence for the existence of an active αβ unit.

N-terminal sequences

The N-terminal sequences of the α and β subunits of the thermophilic *Bacillus* strain RAPc8 nitrile hydratase are shown in Fig. 4, aligned with sequences from a number of bacterial enzymes. The high level of homology in all known bacterial β subunit N-terminal sequences implies a critical structural or functional role. The recently reported 0.265-nm resolution crystal structure of the *Rhodococcus* R312 nitrile hydratase (Huang et al. 1997) shows this 30-residue sequence to wrap around the α subunit. With the catalytic Fe^{III} atom positioned at the subunit interface, the stabilization (and possibly orientation) of the dimer interaction is clearly an important facility. Important as this may be, the extraordinary level of sequence identity (and conservation in nonidentical positions) across β subunits from phylogenetically distant microorganisms is not fully explained. While this level of homology might imply a parallel, functionally directed evolutionary origin, the possibility of horizontal gene transfer at a later point in the evolutionary time-scale should not be discounted.

The complete absence of homology in the N-terminal region of the α subunits suggests that little structural or functional importance is attributed to this region. However, the *Rhodococcus* R312 nitrile hydratase crystal structure shows the N^{α} peptide to be involved in binding of the β subunit core, in a manner reciprocal to the N^{β} peptide. Since both sequences have an apparently similar structural role but neither contributes to active site structure, the diver-

α Subunit

	Residue number 1								10									20					
R. rhodochrous J1-L[1]		M	T	A	H	N	P	V	Q	G	T	L	P	R	S	N	E	E	I	A	A	R	V	K	A	M
R. rhodochrous J1-H[1]									M	S	E	H	V	N	K	Y	T	E	Y	E	A	R	T	K	A	I
R. erythropolis[2]	M	S	V	T	I	D	H	T	T	E	N	A	A	P	A	Q	A	P	V	S	D	G	A	W	A	L
P. chlororaphis B23[3]		M	S	T	S	I	S	T	T	A	T	P	S	T	P	G	.	.	.	.	E	R	A	W	A	L
Rhodococcus sp. N-774[4]	M	S	V	T	I	D	H	T	T	E	N	A	A	P	A	Q	A	P	V	S	D	R	A	W	A	L
Rhodococcus sp. R312[3]							H	T	T	E	N	A	A	P	A	Q	A	P	V	S	D	R	A	W	A	L
Pseudomonas putida[5]	Q	S	H	T	H	D	H	H	H	D	G	Y	Q	A	P	P	E	D	I	A	L	R	V	K	A	L
Pseudonocardia thermophila[6]			M	T	E	N	I	L	R	K	S	D	E	E	I	Q	K	E	I	T	A	R	V	K	A	L
Bacillus sp. RAPc8		M	K	M	M	D	A	N	E	I	I	S	F	I	Q	N	S	K	K	T	T					

β Subunit

	Residue number 1								10									20							
R. rhodochrous J1-L[1]	M	D	G	I	H	D	L	G	G	R	A	G	L	G	P	I	K	P	E	S	D	.	.	.	.
R. rhodochrous J1-H[1]	M	D	G	I	H	D	T	G	G	M	T	G	Y	G	P	V	P	Y	Q	K	D	.	.	.	.
R. erythropolis[2]	M	D	G	V	H	D	L	A	G	V	Q	G	F	G	K	V	P	H	S	V	N	A	.	D	I
P. chlororaphis B23[3]	M	D	G	F	H	D	L	G	G	F	Q	G	F	G	K	V	P	H	T	I	N	S	L	S	Y
Rhodococcus sp. N-774[4]	M	D	G	V	H	D	L	A	G	V	Q	G	F	G	K	V	P	H	T	V	N	A	.	D	I
Rhodococcus sp. R312[3]	M	D	G	V	H	D	L	A	G	V	Q	G	F	G	K	V	P	H	T	V	N				
Pseudomonas putida[5]	M	N	G	I	H	D	T	G	G	A	H	G	Y	G	P	V	Y	R	E	P	N	.	.	.	.
Pseudonocardia thermophila[6]	M	N	G	V	Y	D	V	G	G	T	D	G	L	G	P	I	N	R	P	A	D	.	.	.	.
Bacillus sp. RAPc8	M	N	G	I	H	D	V	G	G	M	D	G	F	G	K	V	M	Y	V	K					

Fig. 4. N-terminal sequence alignments of α and β subunit peptide. Data from: [1]Kobayashi et al. (1991); [2]Duran et al. (1992); [3]Nagasawa et al. (1991); [4]Ikehata et al. (1989); [5]Payne et al. (1997); [6]Yamaki et al. (1997). Alignments after Huang et al. (1997)

gence in apparent evolution of the two sequences remains an anomaly.

pH optimum

Nitrile hydratase activity was determined at a range of pH values (Fig. 5). The optimum pH for activity was 7.0, with 50% activity levels at 5.1 and 8.7. The virtually quantitative loss of activity below pH 5 may reflect either a significantly reduced level of enzyme stability at low pH values or a critical change in ionization of active-site substrate-binding residues.

Inhibition

The effects on nitrile hydratase activity of various inhibitors, metal ions, and chelating agents were determined (Table 2). Complete inhibition of activity by heavy metals and iodoacetamide is consistent with the presence of free thiol residues in the enzyme active site. No change in activity was detected in the presence of 10 mM ethylenediaminetetraacetic acid (EDTA). While this result might argue for the absence of a metal ion in the active site, the inaccessibility of the Fe^{3+} or Co^{2+} ions of other nitrile hydratases (Nagasawa et al. 1991; Nagasawa and Yamada 1987) suggests that chelation data are not a reliable determinant of active-site composition.

Reaction kinetics and substrate specificity

The nitrile-hydratase-catalyzed conversion of nitriles to the corresponding amides was determined quantitatively by gas chromatography. All three aliphatic substrates studied in detail (acetonitrile, valeronitrile, and acrylonitrile) exhibited simple Michaelis-Menten kinetics (acetonitrile and valeronitrile shown in Fig. 6), yielding K_m values of approximately 9 mM, 234 mM, and 11 mM, respectively. Assuming a single active site, turnover numbers (k_{cat}) for the three substrates were approximately 746, 4580, and 484 s^{-1}, respectively, at theoretically saturating concentrations of substrate and a reaction temperature of 50°C. By analogy with other $\alpha_2\beta_2$ nitrile hydratases, the presence of two active centers [one per αβ pair (Huang et al. 1997)] might be inferred.

Bacillus strain RAPc8 nitrile hydratase exhibited a relatively broad substrate specificity (Table 3), although apparently restricted to aliphatic nitriles. Within the limits of detection of the assay system, no reaction with the aromatic substrates benzonitrile or benzylcyanide

Table 2. Effect of inhibitors and metal ions on nitrile hydratase activity

Reagent	Concentration (mM)	% Residual activity
Cu^{2+}	1	0
Ag^{+}	1	0
Iodoacetamide	1	0
Na_2EDTA	10	100
Zn^{2+}	1	110
Mg^{2+}	1	116
Mn^{2+}	1	41
Fe^{2+}	1	112
Fe^{3+}	1	118
Co^{2+}	1	114
PQQ	1	116

EDTA, ethylenediaminetetraacetic acid; PQQ, pyrroloquinoline quinone

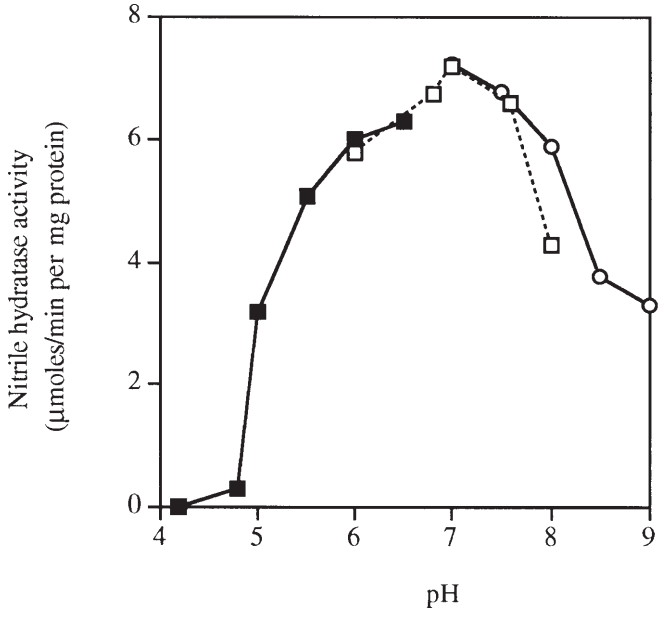


Fig. 5. pH-activity profile. *Closed squares*, Citrate-Na_2HPO_4 buffer; *open squares*, NaH_2PO_4/Na_2HPO_4 buffer; *circles*, Tris-Cl buffer

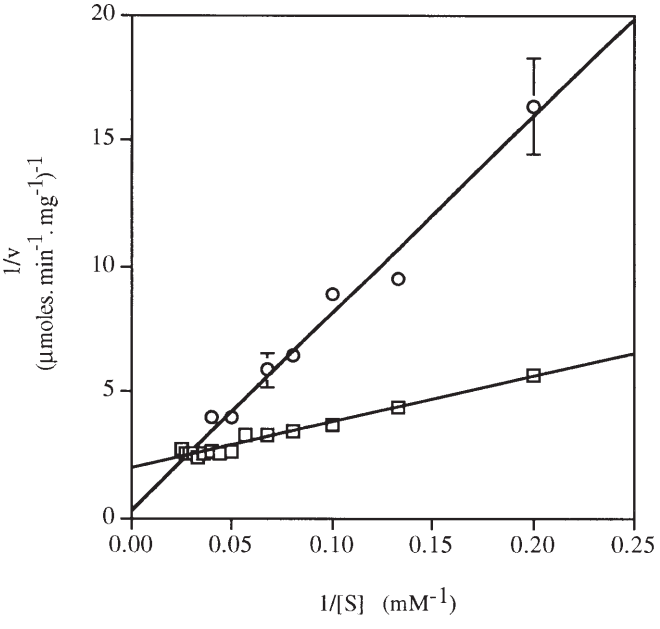
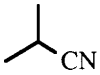
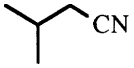
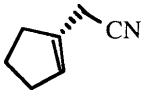
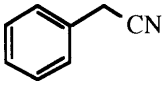
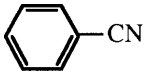
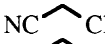
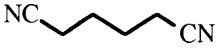


Fig. 6. Lineweaver-Burk plots for acetonitrile (*squares*) and valeronitrile (*circles*) conversions. *v*, velocity; [*S*], substrate concentration

Table 3. Substrate specificities of bacterial nitrile hydratases

Substrate	Sttructure	Relative activity[a,b] (%) for:					
		Bacillus RAPc8	*C. nitrophilus*	*Ps. chlororaphis*	*R. rhodochrous* J1	*R. erythropolis*	*Corynebacterium* sp.
Acetonitrile	CH_3-CN	100	99	2	100	5	6
Chloroacetonitrile	Cl-CH_2-CN	43	–	31	98	2	23
Acrylonitrile		67	100	81	79	33	19
Propionitrile		32	96	100	71	100	23
Methacrylonitrile		64	53	15	14	3	48
Butyronitrile		69	49	77	58	13	46
Isobutyronitrile		59	39	0.1	<1	6	16
Valeronitrile		108	–	3	<1	2	100
Isovaleronitrile		56	–	0	0	–	59
cis,trans-Crotonitrile		50 32	58	0	78	1	10
cis,trans-Cyclo-penteneacetonitrile		70 56	–	–	–	–	–
Benzyl cyanide		0	–	0	<1	0	0.9
Benzonitrile		0	10	0	<1	0	0.9
Malononitrile		7	–	–	–	0	2
Glutaronitrile		32	–	–	–	–	–
Adiponitrile		29	–	–	–	2	–

[a] Relative activity was determined by assuming linear kinetics over a hydrolysis period of 20 min (under standard conditions), with residual substrate determined by gas chromatography analysis. When the formation of amides or the consumption of nitriles was not detected under standard assay conditions, even after the supplementation of enzyme and extended incubation periods, the relative activity was assumed to be zero. –, data not available.

[b] Data from Amarant et al. (1989) (*C. nitrilophilus*), Nagasawa and Yamada (1987) (*P. chlororaphis*), Nagasawa et al. (1991) (*R. rhodochrous* J1), Langdahl et al. (1996) (*R. erythropolis*), and Tani et al. (1989) (*Corynebacterium* sp.).

was detected. Within the aliphatic nitriles, a broader substrate specificity than those of most mesophilic nitrile hydratases was evident, most notably in the relatively high activities on branched chain substrates (methacrylonitrile, isobutyronitrile, and isovaleronitrile) and the cyclic monounsaturated cyclopentene acetonitrile. The hydrolysis of linear, branched, and cyclic unsaturated but not aromatic nitriles suggests that the hydrophobicity of the substrate-binding pocket is not the primary determinant of specificity.

Temperature–activity relationships

Initial rate data obtained over a range of temperatures (Fig. 7) gave an apparent temperature optimum of 60°C with virtually instantaneous inactivation at 70°C. These data, plotted in Arrhenius format (not shown), gave a linear Arrhenius profile in the 30°–60°C range, from which an activation energy of 33 kJ.mole^{-1} was calculated. Although this value is significantly lower than that reported for the mesophilic *Arthrobacter* J-1 nitrile hydratase(Asano et al.

1982), the paucity of comparative data does not allow any valid conclusions to be drawn.

Thermostability

Purified nitrile hydratase was incubated at the various temperatures, sampled periodically, and assayed under standard reaction conditions. The results (Table 4) demonstrate that the thermostability of *Bacillus* strain RAPc8 nitrile hydratase is compatible with the optimum growth temperature of the organism. This enzyme is also considerably more thermostable than most mesophilic nitrile hydratases (Table 5) and significantly more stable than one of the other known thermophilic homologues (Cramp et al. 1997; Yamaki et al. 1997). Nevertheless, caution must be exercised in such comparisons because of variations in incubation conditions and the addition, in some of experiments quoted (Nagasawa et al. 1991; Yamaki et al. 1997), of specific stabilizing reagents such as *n*-butyric acid and sodium valerate.

Conclusions

It is evident that thermostable nitrile hydratase from the thermophilic *Bacillus* strain RAPc8 is closely related to mesophilic nitrile hydratases. In particular, the similar physical properties of the mesophilic and thermophilic enzymes (e.g., subunit size and quaternary composition) together with the extreme conservation of the β-subunit N-terminal sequence, argue for a common genetic origin rather than convergent evolution.

The thermostability of the *Bacillus* strain RAPc8 nitrile hydratase is consistent with the degree of thermophily of the parent organism, showing marginal stability at temperatures around the growth optimum (65°C). Unlike extracellular enzymes, which are often stable at temperatures 10 to 30 degrees higher than the temperature optimum of the source organism (Daniel et al. 1996), the upper limit of stability of the inducible intracellular nitrile hydratase is probably determined by the need to recycle the protein rapidly on depletion of the substrate/inducer.

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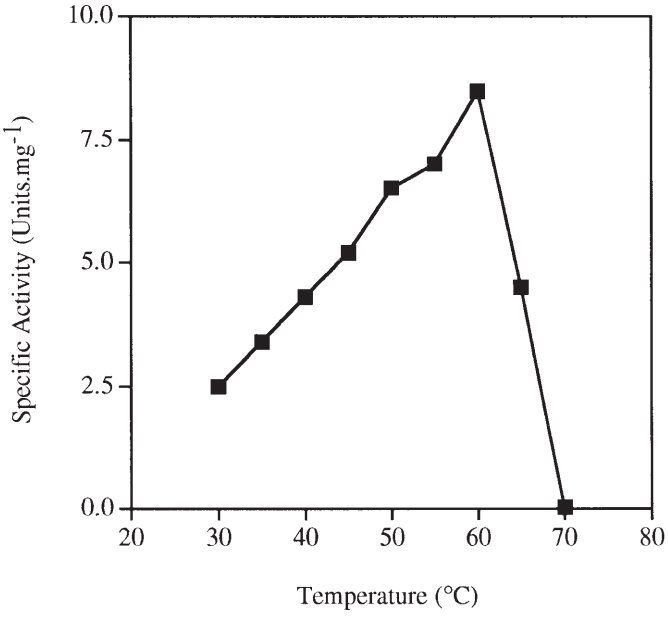

Fig. 7. Temperature-activity profile for *Bacillus* strain RAPc8 nitrile hydratase. Error values for these data were <1%

Table 4. Thermostability of *Bacillus* strain RAPc8

Temperature (°C)	Half-life of activity
60	<20 minutes
50	2.5 hours
37	45 hours
20	80 hours
4	>1 month

Table 5. Thermostability of bacterial nitrile hydratases

Organism	Half-life of activity[a]
Corynebacterium pseudodiphteriticum	65 min at 20°C (Li et al. 1992)
Pseudomonas chlororaphis B23	11 min at 30°C (Nagasawa et al. 1987)
Rhodococcus rhodochrous J1	58 min at 60°C[b] (Nagasawa et al. 1991)
Corynebacterium sp. C5	7.5 min at 45°C[c] (Tani et al. 1989)
Bacillus pallidus	7.3 min at 60°C[d] (Cramp et al. 1997)
Pseudomonas putida	27 min at 50°C (Payne et al. 1997)
Pseudonocardia thermophila	13 h at 60°C[e] (Yamaki et al. 1997)

[a] Recalculated from authors' data assuming logarithmic activity loss.
[b] Stabilized by presence of 44 mM *n*-butyric acid.
[c] Stabilized by addition of 30 mM Na.isovalerate.
[d] Activity in crude cell-free extract.
[e] Stabilized by addition of 34 mM *n*-butyric acid.

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