Extremophiles (1998) 2:207–216

REVIEW

Don Cowan · Rebecca Cramp · Rui Pereira
Dan Graham · Qadreyah Almatawah

Biochemistry and biotechnology of mesophilic and thermophilic nitrile metabolizing enzymes

Received: January 22, 1998 / Accepted: February 16, 1998

Abstract Mesophilic nitrile-degrading enzymes are widely dispersed in the Bacteria and lower orders of the eukaryotic kingdom. Two distinct enzyme systems, a nitrilase catalyzing the direct conversion of nitriles to carboxylic acids and separate but cotranscribed nitrile hydratase and amidase activities, are now well known. Nitrile hydratases are metalloenzymes, incorporating Fe^{III} or Co^{II} ions in thiolate ligand networks where they function as Lewis acids. In comparison, nitrilases are thiol-enzymes and the two enzyme groups have little or no apparent sequence or structural homology. The hydratases typically exist as αβ dimers or tetramers in which the α- and β-subunits are similar in size but otherwise unrelated. Nitrilases however, are usually found as homomultimers with as many as 16 subunits. Until recently, the two nitrile-degrading enzyme classes were clearly separated by functional differences, the nitrile hydratases being aliphatic substrate specific and lacking stereoselectivity, whereas the nitrilases are enantioselective and aromatic substrate specific. The recent discovery of novel enzymes in both classes (including thermophilic representatives) has blurred these functional distinctions. Purified mesophilic nitrile-degrading enzymes are typically thermolabile in buffered solution, rarely withstanding exposure to temperatures above 50°C without rapid inactivation. However, operational thermostability is often increased by addition of aliphatic acids or by use of immobilized whole cells. Low molecular stability has frequently been cited as a reason for the limited industrial application of "nitrilases"; such statements notwithstanding, these enzymes have been successfully applied for more than a decade to the kiloton production of acrylamide and more recently to the smaller-scale production of nicotinic acid, R-(−)-mandelic acid and S-(+)-ibuprofen. There is also a rapidly growing catalog of other potentially useful conversions of complex nitriles in which the regioselectivity of the enzyme coupled with the ability to achieve high conversion efficiencies without detriment to other sensitive functionalities is a distinct process advantage.

Key words Mesophile · Thermophile · Nitrile hydratase · Nitrilase · Nitrile · Thermostable · Biotransformation

Communicated by J. Wiegel

D. Cowan (✉) · R. Cramp · R. Pereira · D. Graham · Q. Almatawah
Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, U.K.
Tel. +44-171-504-2246; Fax +44-171-380-7193
e-mail: don.cowan@ucl.ac.uk

Distribution of nitrile-degrading enzyme systems

Nitrile (organocyanide) metabolizing enzyme systems are widely if relatively infrequently distributed through two of the three domains. The existence of nitrilase activity in only 3 of 21 plant families (Gramineae, Cruciferae, and Musaceae) (Thimann and Mahadevan 1964a) and in a limited number of fungal genera (e.g., *Fusarium*, *Aspergillus*, *Penicillium*) (Harper 1977a) indicates the relative rarity of this activity. Nitrile-degrading activities are apparently more frequent in Bacteria (see Tables 1 and 2), although it is impossible to assess the true distribution frequency accurately without access to extensive screening data. It is probable that entire groups of microorganisms have never been screened while others may have yielded negative and hence unreported results. A determination of nitrile-degrading activities in catalogs of thermophilic *Bacillus* and *Thermus* strains in the authors' laboratory (Cramp, Pereira, and Cowan, unpublished results) gave less than 10% and 0% hits respectively, while Duran reported only two positive signals in a Southern hybridization analysis of 31 bacterial species from 17 genera (Duran et al. 1993). To the author's knowledge, no reports currently exist of nitrile-degrading activity in the Archaea. Furthermore, none of the extant complete or partial archaeal genome sequences (Bult et al. 1996; Klenk et al. 1997; Sensen et al. 1996; Smith et al. 1997; Stein et al. 1996) show any significant homology to bacterial or eukaryotic nitrile hydratase or nitrilase gene sequences.

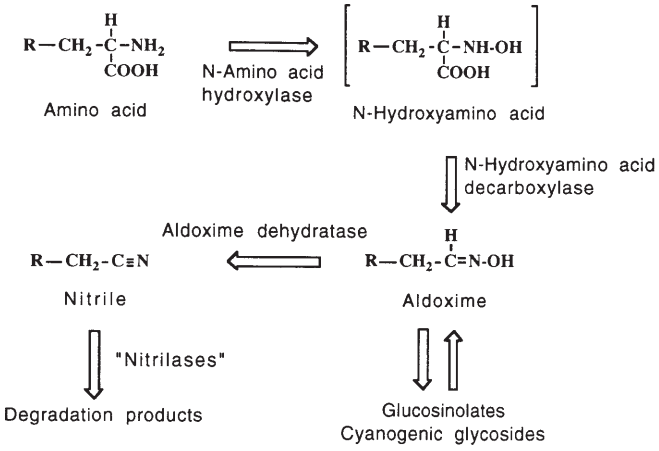

Fig. 1. Role of nitrile-degrading enzymes in plant nitrogen turnover

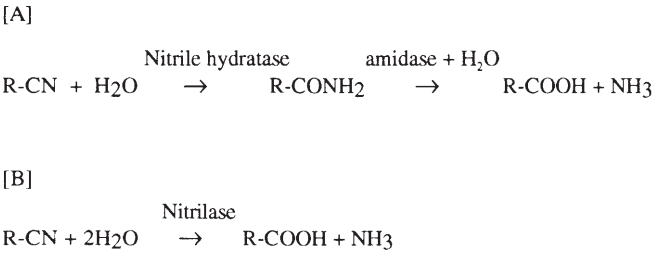

Fig. 2. Metabolism of nitriles: [*A*] nitrile hydratase/amidase; [*B*] Nitrilase

The apparent distribution of bacterial nitrile-degrading enzymes (e.g., *Rhodococcus*, *Nocardia*, *Brevibacterium*, *Pseudomonas*, and *Corynebacterium*; see Tables 1 and 2) may owe more to screening technologies than to molecular evolution or gene transfer. The most common screening protocols involve enrichment isolation in nitrile-supplemented C- or N-limited medium, inevitably skewing positive clones to selected groups of resilient and readily culturable microorganisms.

The physiological role of nitrile-degrading enzymes in bacteria is by no means clear. In plants, such activities are implicated in nutrient mobilization, particularly in the degradation of glucosinolates (Bestwick et al. 1993). It has also been suggested that nitrile-degrading enzymes form components of complex pathways controlling both the production and degradation of cyanogenic glycosides and related compounds and where aldoximes are key intermediates (Fig. 1). While such a role has yet to be established in bacterial systems, it is noted that some of the upstream enzyme activities, in particular the key enzyme aldoxime dehydratase, have been detected in *Pseudomonas* (Milborrow 1963).

Biochemical properties of mesophilic nitrile-degrading enzymes

The bacterial conversion of nitriles involves two well-established and quite separate enzyme systems (Fig. 2). The nitrilases, which frequently show a strong preference for aromatic substrates, convert nitriles directly to the corresponding acid. Alternatively, conversion via the amide intermediate involves two separately encoded but coordinately expressed enzyme activities, nitrile hydratase and amidase. The adjacent (and sometimes overlapping) positioning of the open reading frames encoding the nitrile hydratase α- and β-subunits and the amidase monomer suggest that these genes may be cotranscribed as a single mRNA (Duran et al. 1993; Nishiyama et al. 1991). Nitrilases and some nitrile hydratases are inducible proteins. Analysis of a limited number of nitrilase and nitrile hydratase operons suggests that both upstream (Komeda et al. 1996a,b) and downstream (Hashimoto et al. 1994; Nishiyama et al. 1991) open reading frames are involved in positive transcriptional regulation.

Bacterial nitrile hydratases show considerable sequence and structural homology. For example, the α- and β-subunit sequence homologies identified between *Pseudomonas chlororaphis* B23 and *Rhodococcus* sp. N-774 were 63% and 58%, respectively (Ikehata et al. 1989; Nishiyama et al. 1991). Comparisons of shorter N-terminal amino acid sequences show high levels of sequence homology in the β-subunit (Fig. 3) but no significant homology in the α-subunit. The explanation for the latter has only become apparent with the publication of the first detailed X-ray crystallographic structure of a nitrile hydratase. Although the conserved N-terminal sequence of the β-subunit was buried in the center of the αβ heterodimeric interface (Huang et al. 1997), the N-terminal sequence of the α-subunit was not buried, although still involved in αβ dimer stabilization by wrapping across the surface of the β-subunit.

Alignments of nitrilase monomer amino acid sequences (Levy-Schil et al. 1995) from *Comamonas*, *Klebsiella*, *Alcaligenes*, and two *Rhodococcus* strains show only 13% identity, but with some residues conserved across eukaryotic nitrilase and cyanide hydratase sequences. One conserved cysteine has been implicated in catalytic activity by site-directed mutagenesis (Kobayashi et al. 1992a).

The native quaternary structures of bacterial nitrile hydratases are highly variable (Table 1), ranging from 30 to 505 kDa. However, with the exception of the homotetrameric *P. chlororaphis* enzyme (Nagasawa et al. 1987), all other known bacterial nitrile hydratases are αβ heteromultimers, typically dimers or tetramers. There is also remarkable consistency in the sizes of the subunits: α, 24–28 kDa; β, 25–39 kDa. Nitrilases, by comparison, are homomultimers of approximately 38- to 76-kDa subunits with native compositions ranging from α_2 to α_{16}.

Both nitrile-degrading enzyme systems contain thiolate ligands at the active site. Inhibitor studies typically show sensitivity to sulfhydryl reagents, oxidizing agents, and heavy metals, but little or no reaction to serine inhibitors or, surprisingly, metal chelating agents. This result is inconsistent with the accepted view that most if not all nitrile

Table 1. Molecular and functional characteristics of mesophilic nitrile hydratases

Microorganism	Native MW (kDa)	Subunit MW (kDa)	Active center	T_{opt} (°C)	pH optimum	Substrate preference	Photoactivation	Production	Coding of structural genes	Reference
Agrobacterium tumefaciens		α 28	Fe^{III} Co	25	6.5–9.5		ND	Inducible		Bauer et al. (1994)
Arthrobacter sp. J-1	420	α 24 β 27	-SH			Aliphatic	ND	Inducible		Asano et al. (1982)
Corynebacterium nitrophilus					6–8	Aliphatic, dinitriles, aromatic	ND			Amarant et al. (1989)
C. pseudodiphtheriticum ZBB-41	80	α 25 β 28	Fe^{III}			Aliphatic	ND			Li et al. (1992)
Corynebacterium sp. C5	61	26.9	PQQ? Fe^{III}			Aliphatic, dinitriles, cyclic aliphatic	ND			Yamamoto et al. (1992b)
Pseudomonas chlororaphis	100	α 25 β 25	Fe^{III}	20	6.0–7.5		–	Inducible	αβ	Nagasawa et al. (1987); Yamada et al. (1986)
Pseudomonas putida	54, 95	α 23 β 24.1	Co^{II}							[Payne et al. (1997) #162]
Rhodococcus equi SHB-121	30	30				Aliphatic, cyclic	ND			Gilligan et al. (1993); Martinkova et al. (1995)
R. erythropolis BL1						Aliphatic, aromatic	ND	Inducible	αβ	Duran et al. (1993)
R. rhodochrous J1	505	α 26 β 29	Co	35–40	6.0–8.5	Aliphatic, aromatic	–	Inducible	βα	Kobayashi et al. (1991); Komeda et al. (1996c); Nagasawa et al. (1991a,b)
Rhodococcus sp. N-774[a]	70	α 27 β 27	Fe^{III}	35	7.0–8.5	Aliphatic	+	Constitutive	αβ	Endo and Watanabe (1989); Hashimoto et al. (1994)
Rhodococcus sp. N-771[a]	70	α 27 β 27	Fe^{III}, PQQ			Aliphatic	+	Inducible	αβ	Ikehata et al. (1989); Yamada and Kobayashi (1996)
Rhodococcus sp. R312[a,b]	95	α β	Fe^{III}	35	7–8.5	Aliphatic, cyclic	+		αβ	Nagasawa et al. (1986)

PPQ, pyrroloquinoline quinone; ND, not determined.
[a] Sequences identical.
[b] Originally designated as *Brevibacterium* R312.

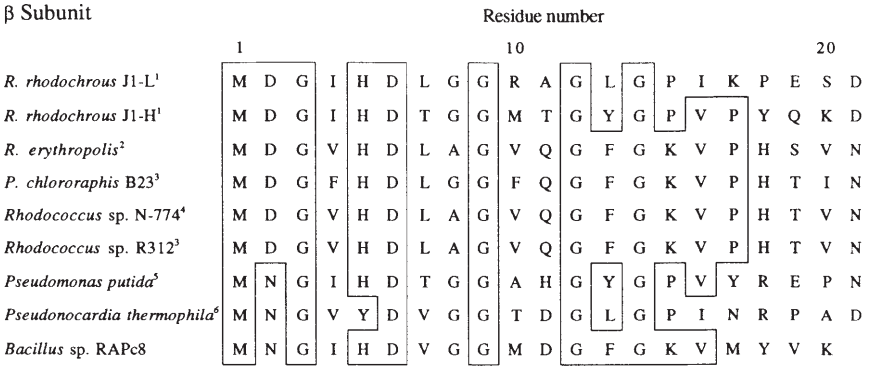

Fig. 3. Alignment of N-terminal amino acid sequences from mesophilic and thermophilic nitrile hydratase β-subunits. Data from: [1][Kobayashi, 1991 #105], [2][Duran, 1992 #98], [3][Nagasawa, 1991 #118], [4][Ikehata, 1989 #136], [5][Payne, 1997 #162], [6][Yamaki, 1997 #166]. Alignments after [Huang, 1997 #164]

Data from: [1] [Kobayashi, 1991 #105], [2] [Duran, 1992 #98], [3] [Nagasawa, 1991 #118], [4] [Ikehata, 1989 #136], [5] [Payne, 1997 #162], [6] [Yamaki, 1997 #166]. Alignments after [Huang, 1997 #164].

hydratases contain either low-spin nonheme Fe^{III} or noncorrinoid Co^{II} cofactors (Huang et al. 1997). Suggestions that *P. chlororaphis* B23 and *Brevibacterium* R312 nitrile hydratases contained pyrroloquinoline quinone (PQQ) prosthetic groups (Nagasawa and Yamada 1987; Sugiura et al. 1988) have more recently been discarded (see Kopf et al. 1996). The *Rhodococcus* R312 (*Brevibacterium* R312 has been reclassified as *Rhodococcus* R312) nitrile hydratase binds two atoms of Fe within the α-subunit, using three cysteine thiolates and two main chain amide nitrogen atoms as ligands. A subset of the low-spin nonheme iron-containing nitrile hydratases show an interesting photoactivation effect (Nagamune et al. 1990). Cells of *Rhodococcus* sp. N771 grown in the dark show no nitrile hydratase activity, but become catalytically active after light irradiation. Using Mossbauer, electron spin resonance (ESR), and magnetic susceptibility measurements (Honda et al. 1992), the oxidation of Fe^{II} to an Fe^{III} state has been implicated in photoactivation. Most recently, it has been shown that the reduced Fe of the inactive enzyme binds NO (nitric oxide) in stoichiometric quantities (Bonnet et al. 1997) and that NO is released on photoactivation. The importance of NO as a neurotransmitter and physiological regulator in higher eukaryotes notwithstanding, the physiological significance of this process in *Rhodococcus* [and possibly in several other bacterial nitrile hydratases (Bonnet et al. 1997)] is not currently obvious.

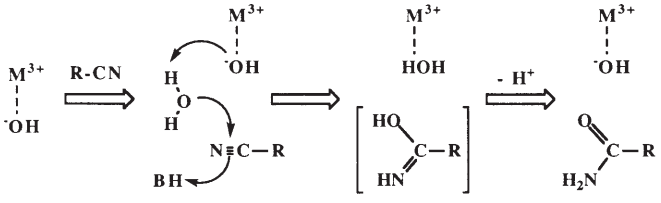

Fig. 4. Proposed mechanism for nitrile hydratase catalysis. (Redrawn from Huang et al. 1997)

The catalytic mechanism of iron-containing nitrile hydratases has been suggested to involve the Fe^{III} centre as a Lewis acid (Huang et al. 1997), either directly or indirectly activating the nitrile for hydration (Fig. 4). Mechanisms involving direct coordination of the nitrile nitrogen to the metal ion or attack on the nitrile by an iron-coordinated hydroxyl group are thought to be less likely, because trivalent metal ions typically show slow rates of ligand exchange (Huang et al. 1997). Indirect attack via a water molecule (as shown in Fig. 4) does not require ligand exchange and is consistent with the role of the Fe^{III} as a Lewis acid. Nucleophilic attack on the nitrile by an active site thiolate as proposed by Kopf et al. (1996) is probably not significant in *Rhodococcus* R312 because the crystal structure shows all thiolate ligands to be strongly coordinated to the Fe center (Huang et al. 1997).

The catalytic mechanisms of the nitrilases are less well established. A single conserved cysteine residue in a number of nitrilase sequences (Levy-Schil et al. 1995) and inhibitor studies (Kobayashi et al. 1989) are consistent with

Table 2. Molecular and functional characteristics of mesophilic nitrilases

Microorganism	Native and (subunit) MW (kDa)	Optimum pH	T_{opt} (°C)	Substrate specificity	pI	Inducible	Gene cloned	Other characteristic	References
Acinetobacter sp. AK226	580 (43)	8	50	Aliphatic, aromatic, heterocyclic	3.99	No	?	Enantioselective	Yamamoto and Komatsu (1991)
Arthrobacter sp. strain J-1									
A	30	8.5	40	Aromatic and heterocyclic nitriles	4.95	Yes	?	Nitrile hydratase and	Bandyopadhyay et al. (1986)
B	23	7.5	30	Aromatic and heterocyclic nitriles	4.8	Yes	?	amidase activity	
Alcaligenes faecalis JM3	275 (44)	7.5	45	Arylacetonitriles	4.9	Yes	Yes	Arylacetonitrilase	Kobayashi et al. (1993); Nagasawa et al. (1990)
A. faecalis ATCC 8750	460 (32)	7.5	40–45	Substituted aliphatic nitriles	?	Yes	?	Arylacetonitrilase and enantioselective activities	Yamamoto et al. (1992a)
Comamonas testosteroni	(38.7)			Dinitriles			Yes	High activity on adiponitrile	Levy-Schil et al. (1995)
Fusarium solani	620 (76)	7.8–9.1		Aromatic and heterocyclic nitriles	4.19	Yes	?		Harper (1977a)
F. oxysporum f. sp. *melonis*	550 (37)	6.0–11	40	Aromatic and heterocyclic nitriles	<4	Yes	?		Goldlust and Bohak (1989)
Nocardia sp. NCIB 11216	560 (45)	8		Aromatic nitriles	4.22	Yes	?		Harper (1977b)
Nocardia sp. NCIB 11215	560 (46)	7.0–7.5		Aromatic and heterocyclic nitriles	4.35	Yes	?		Harper (1985)
Rhodococcus rhodochrous J1	78 (41.5)	7.6	45	Aromatic and heterocyclic nitriles	5.6	Yes	Yes	Regiospecific	Kobayashi et al. (1992a, 1989)
Rhodococcus ATCC 39484	560 (40)	7.5	40	Aromatic and heterocyclic nitriles	?	Yes	?	Enantioselective	Stevenson et al. (1992)
R. rhodochrous K22	650 (41)	5.5	50	Aliphatic nitriles	?	Yes	Yes		Kobayashi et al. (1992b)

Table 3. Substrate specificity of *Bacillus pallidus* DAC521 nitrilase

Substrate	Relative activity (%)
Aliphatic	
Saturated	
Acetonitrile	3
Chloroacetonitrile	10
Trichloroacetonitrile	0
Propionitrile	0
Butyronitrile	19
Isobutyronitrile	0
4-Chlorobutyronitrile	110
Valeronitrile	43
Isovaleronitrile	0
Saturated dinitrile	
Malononitrile	0
Succinonitrile	0
Glutaronitrile	11
Adiponitrile	14
Iminodiacetonitrile	0
3,3-Iminodipropionitrile	0
Unsaturated	
Acrylonitrile	9
Methacrylonitrile	2
Allylcyanide	20
Crotononitrile	80
cis-2-pentenenitrile	0
trans-3-pentenenitrile	23
3-Aminopropionitrile fumarate	0
Unsaturated dinitrile	
Fumaronitrile	16
Mucononitrile	2
Heterocyclic	
2-Cyanopyridine	92
3-Cyanopyridine	135
4-Cyanopyridine	233
1-Cyclopentene acetonitrile	0
1-Cyanoacetylpiperidine	0
Aromatic	
Benzonitrile	100
2-Aminobenzonitrile	0
3-Aminobenzonitrile	24
4-Aminobenzonitrile	3
2-Chlorobenzonitrile	3
3-Chlorobenzonitrile	50
4-Chlorobenzonitrile	192
2-Hydroxybenzonitrile	0
3-Hydroxybenzonitrile	57
4-Hydroxybenzonitrile	29
2-Fluorobenzonitrile	27
3-Fluorobenzonitrile	157
4-Fluorobenzonitrile	156
2-Nitrobenzonitrile	2
3-Nitrobenzonitrile	21
4-Nitrobenzonitrile	23
o-Tolunitrile	44
m-Tolunitrile	30
p-Tolunitrile	77
3-Chloro-4-fluorobenzonitrile	95
3,4,Dibromo-4-*OH*-benzonitrile	0
Phenylacetonitrile	1
4-Aminobenzylcyanide	0
Mandelonitrile	0
2-(Methoxyphenyl)acetonitrile	6
3-(Benzylamino)propionitrile	0
Cinnamonitrile	6
Benzylidenemalononitrile	0

The relative activity of the compounds shown was measured at 50°C and pH 7.0 with a substrate concentration of 100 mM in the presence of 100 mM urea. Relative activities are expressed relative to benzonitrile (100%).

nucleophilic attack on the nitrile carbon by an activated thiol residue (Harper 1977; Thimann and Mahadevan 1964b). Metal ions have not been implicated in the catalytic process. The role of an active site thiolate nucleophile is further supported by the recent report of the generation of nitrilase activity in the cysteine protease papain by a Gln19Glu mutation in the active site (Dufour et al. 1995).

Tables 1 and 2 show the general functional properties of bacterial nitrile hydratases and nitrilases. The relatively low temperature optima of both groups of enzymes reflect the growth optima of the source organisms and the intracellular nature of the enzymes. In some cases, these values include in vitro stabilization by compounds such as alkanoic acids (Nagasawa et al. 1987). Nevertheless, there is a consensus that the Co-containing hydratases are generally more thermostable than the iron-containing enzymes (Payne et al. 1997). The neutral-to-alkaline pH optima are probably as much influenced by the acid-dependent hydrolysis of organocyanides as by pH-dependent changes in active site residue (thiolate) ionization.

The specificities of mesophilic nitrile hydratases and nitrilases are only cursorily shown in Tables 1 and 2, the reader being directed to several excellent reviews (Meth-Cohn and Wang 1997a,b; Sugai et al. 1997a,b) and to the original papers for more detailed information. What is clear, however, is that the early dogma that "nitrile hydratases are aliphatic substrate specific and lack enantioselectivity" while "nitrilases are aromatic substrate specific and enantioselective" has been severely shaken by the discovery of nitrile hydratases capable of catalyzing the hydration of linear, branched, and cyclic aliphatic nitriles together with substituted and complex aromatic nitriles (Meth-Cohn and Wang 1997a,b), by the demonstration of stereoselectivity in purified nitrile hydratases (as opposed to whole-cell systems where the presence of the amidase may complicate interpretation) (Martinkova et al. 1996) and by the characterization of very broad specificity nitrilases (Almatawah and Cowan, unpublished results) (Table 3).

Biotransformations of nitriles

Probably the major driving force behind studies of nitrile-metabolizing enzymes comes from the potential capacity of these enzymes to perform valuable biotransformations. Nitriles are important intermediates in chemical synthesis, particularly as products of one-, two-, and three-carbon additions to core synthons. Although the chemical conversion of nitriles to amides and acids is facile, relatively harsh reactions conditions (heat, acid, or alkali) are required. Such conditions may be incompatible with the presence of sensitive functionalities such as ester-linked groups. The ability to convert nitriles to amides or acids under very mild conditions (neutral to mildly alkaline pH, ambient temperatures), especially with the advantage of chemo-, regio-, or stereoselectivity, underpins the growing interest in nitrile-metabolizing enzymes.

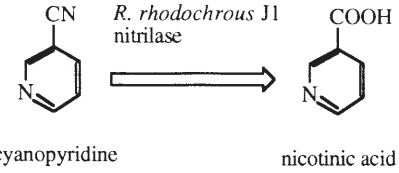

Fig. 5. Biosynthesis of nicotinic acid

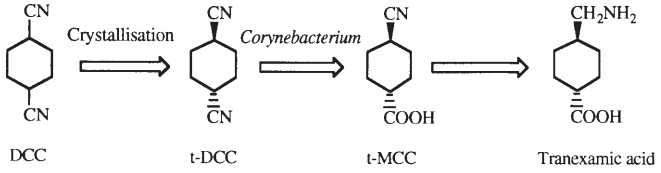

Fig. 7. Bacterial semisynthesis of tranexamic acid

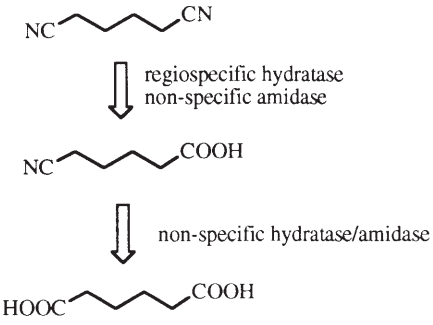

Fig. 6. Conversion of adiponitrile to adipic acid

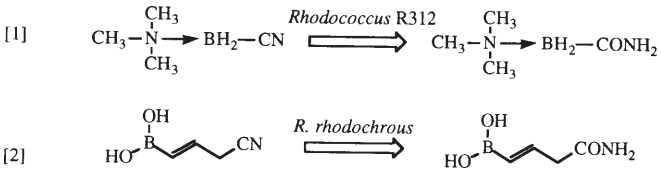

Fig. 8. Biotransformations of boron-containing nitriles

In one aspect, the biotechnology of nitrile biotransformations has already matured. The success of three generations of nitrile-metabolizing bacteria, *Rhodococcus* N-774, *Pseudomonas chlororaphis* B23, and *R. rhodochrous* J1, in the kiloton production of acrylamide (Nagasawa et al. 1993; Yamada and Kobayashi 1996) has unarguably demonstrated the commercial viability of these enzymes. The biosynthesis of nicotinic acid from 3-cyanopyridine (Mathew et al. 1988) (Fig. 5) is also on the verge of large-scale commercialization.

This review is not an appropriate forum for a detailed summary of nitrile biotransformations, but merely attempts to demonstrate the potential scope of "nitrilase" applications by reviewing recent examples.

Example 1: synthesis of cyanovaleric acid and adipic acid

The conversion of adiponitrile to cyanovaleric acid and hence to adipic acid (Fig. 6) provides a synthetic route to the production of intermediates in the formation of the polymers nylon 6 and nylon 6,6. Nitrilases showing high activity on adiponitrile and the cyano-acid intermediate have only recently been identified (Moreau et al. 1994). For example, a *Comamonas testosteroni* nitrilase was reported (Levy-Schil et al. 1995) to have a specific activity for conversion of adiponitrile to cyanovaleric acid (CVA) of 29 μmoles CVA produced/min/mg protein, and activity for the conversion of CVA to adipic acid of approximately 20% of this value. Relative activities on standard aliphatic (propionitrile) and aromatic (benzonitrile) substrates were substantially lower.

Example 2: semisynthesis of tranexamic acid

Tranexamic acid (*trans*-4-aminomethylcyclohexane-1-carboxylic acid) is an antifibrinolytic agent that is synthesized chemically from *cis*, *trans*-cyclohexane-1,4-dimethylcarboxylate (Fig. 7) and obtained as a pure *trans* product by repeated and difficult crystallization from a mixture of *cis* and *trans* products. A simpler separation of the *cis*, *trans*-dicyanocyclohexanes is precluded because of the lack of mononitrile specificity in the subsequent alkaline hydrolysis and conversion of the *trans* to the *cis* isomer. An alternative process for the biological conversion of *trans*-dicyanocyclohexane (t-DCC) to *trans*-4-cyanocyclohexane-1-carboxylic acid (t-MCC) (Yamamoto et al. 1992b) therefore avoids both problems associated with the chemical conversion. Reaction of insoluble t-DCC in aqueous solution (up to 60% by weight) at 4°C with resting cells of *Corynebacterium* C5 gave yields in excess of 99% with no transversion of stereochemical configuration.

Example 3: biotransformation of boron-containing nitriles

The production of boron-containing organic compounds offers a variety of synthetic routes to the generation of new and potentially valuable compounds. For example, the boron-containing amino acid analogs (amine-carboxyboranes) possess a variety of biological activities and have potential application as antineoplastic, antihyperlipidemic, analgesic, and antiarthritic agents (Spielvogel 1988). Such compounds may be chemically synthesized at low yield from amine cyanoboranes in an inert gas atmosphere, but the possibility of bioconversion using nitrilases potentially offers significant advantages (Fig. 8, scheme [1]).

Table 4. Structural and functional properties of thermophilic nitrile-metabolizing enzymes

Activity	Nitrile hydratase	Nitrile hydratase	Nitrile hydratase	Nitrilase
Source	*Bacillus* sp.	*B. pallidus*	*Pseudonocardia thermophila*	*B. pallidus*
Growth T_{opt}	65°C	50°C	55°C	60°C
Enzyme composition	$\alpha_2\beta_2$	$\alpha_2\beta_2$	$\alpha_n\beta_m$	α_{14}
Native and subunit MW (kDa)	110 28, 29	110 27, 29	Unknown 29, 32	600 42.6
Specificity	Very broad: Linear, branched and cyclic aliphatics	Narrow: aliphatic	Unknown	Very broad: aromatic, heterocyclic, branched and cyclic aliphatic
Reference	Pereira et al. (this issue)	Cramp (1998); unpublished	Yamaki et al. (1997)	Almatawah et al., unpublished results

A recent preliminary report (Millais et al. 1997) demonstrating that *Rhodococcus* R312 is probably capable of catalyzing the slow conversion of trimethylamine-cyanoborane to the amide derivative trimethylamine-carboamidoborane suggests that such unusual reactions are worthy of further attention. Similarly, *R. rhodochrous* cells have been shown to rapidly convert nitrile-derivatized alkenylboronic acids to the corresponding amides (Fig. 8, scheme [2]) (Sugai et al. 1997a). Further transformation by intracellular amidase to the acid was slow and relatively low yields were obtained, even after prolonged incubation. Reaction of the amide intermediates with haloalkenes provides a route to the synthesis of derivatized alkenes with conjugated double bonds having defined configurations.

Thermophilic nitrile-metabolizing enzymes

Relatively few examples of thermophilic nitrile-metabolizing enzymes have yet been reported. Detailed publications are limited to nitrile hydratases derived from several moderately thermophilic *Bacillus* species (Cramp et al. 1997; Pereira et al., [this issue]; Takashima et al. 1995, 1996) and a new thermophilic actinomycete, *Pseudonocardia thermophila* (Yamaki et al. 1997). To the author's knowledge, no reports of thermophilic nitrilases have yet been published although one such enzyme has been characterized in detail in the author's laboratory (Almatawah and Cowan, unpublished data).

Some molecular and functional properties of the known thermophilic nitrile-metabolizing enzymes are shown in Table 4. The striking conclusion from these data is that, thermostability apart, the general structural and functional characteristics of these enzymes are remarkably similar to their mesophilic "homologs." This similarity is also evident in the sequence identity of the N-terminal regions of the nitrilases (Almatawah and Cowan, unpublished results) and nitrile hydratase β-subunits (see Fig. 3). This conclusion is consistent with the accepted view that thermophilicity in all Bacteria other than the few deeply branched genera such as the hyperthermophiles *Thermotoga* and *Aquifex* is a relatively recent evolutionary product.

Table 5. Thermal stability values of purified and crude (values in brackets) thermophilic nitrile-metabolizing enzymes

Half-life values at temperature in °C	*Bacillus pallidus* NHase	*Bacillus* strain NHase	*B. pallidus* Nase
30	7.0 h (120 h)	80 h	– (7 d)
37	– (67 h)	45 h	– (3.5 d)
50	– (4.5 h)	2.5 h	8 h (6.5 h)
55	51 m –	–	– –
60	6.8 m (8.2 m)	<20 min	2.5 h (34 m)
70	–	–	<10 min –
80	–	–	<5 min –

The thermostabilities of the thermophile-derived nitrile-metabolizing enzymes (Table 5) are all substantially higher than those observed for homologous enzymes derived from mesophilic organisms. As might be expected, the purified enzymes all show significant stability at temperatures around the optimal growth temperature of the source organisms. The stability of *B. pallidus* nitrile hydratase activity is, however, substantially greater in unbroken cells, indicating that the intracellular environment provided a significant level of extrinsic stabilization.

Many of the mesophilic nitrile-metabolizing enzymes are remarkably unstable, having very short activity half-lives in the growth temperature range of 20°–35°C [e.g., *Corynebacterium pseudodiphtheriticum*, $t_{1/2}$ = 65 min at 20°C (Li et al. 1992); *Pseudomonas chlororaphis* B23, $t_{1/2}$ = 11 min at 30°C (Nagasawa et al. 1987); *Rhodococcus rhodochrous* J1, $t_{1/2}$ = 58 min at 60°C (Nagasawa et al. 1991a,b); *Corynebacterium* sp. C5, $t_{1/2}$ = 7.5 min at 45°C (Tani et al. 1989)]. Furthermore, the values quoted for the *R. rhodochrous* and *Corynebacterium* sp. C5 enzymes are for stabilities in the presence of aliphatic acids (butyrate or valerate), compounds that strongly stabilize some mesophilic nitrile hydratases (Kopf et al. 1996). The relatively high stabilities of the thermophilic nitrile-metabolizing enzymes, their rapid rates of turnover even at suboptimal temperatures (Cramp et al. 1997; Pereira et al., [this issue]) and their broad substrate specificities (Table 6) all contribute to the view that these enzymes may in the future be successfully

Table 6. Comparative specificities of thermophilic *Bacillus* nitrile-metabolizing enzymes

Substrate	Structure	*B. pallidus* NHase	*Bacillus* RAPc8 NHase	*B. pallidus* Nase
Acetonitrile	CH_3CN	93	60	3
Propionitrile	CH_3CH_2CN	30	40	0
Butyronitrile	$CH_3(CH_2)_2CN$	64	55	19
Valeronitrile	$CH_3(CH_2)_3CN$	100	49	43
Acrylonitrile	$CH_2=CHCN$	62	79	9
Crotonitrile	$CH_3CH=CHCN$	38	100	80
Benzonitrile	C_6H_5CN	0	0	100
Cyclopentene-acetonitrile	$C_5H_7CH_2CN$	58	0	0
Malononitrile	$NCCH_2CN$	6	0	0
Glutaronitrile	$NC(CH_2)_3CN$	30	0	11
Adiponitrile	$NC(CH_2)_4CN$	27	0	14

Values are relative activities, normalized to the maximum value.

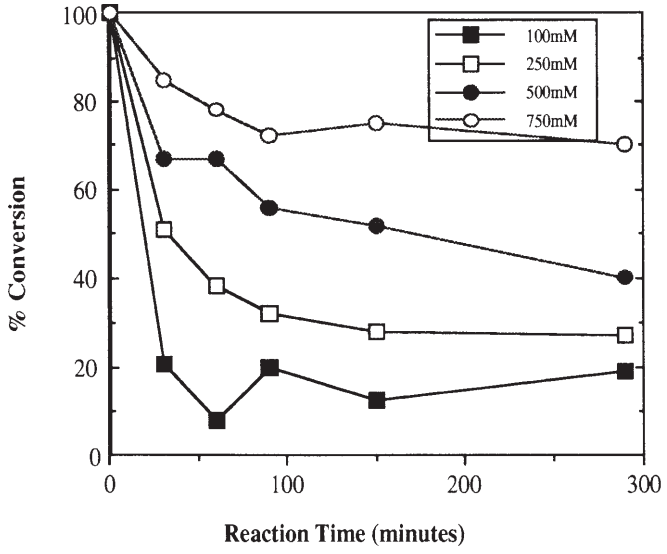

Fig. 9. Biotransformation of acrylonitrile with encapsulated thermophilic *Bacillus* cells

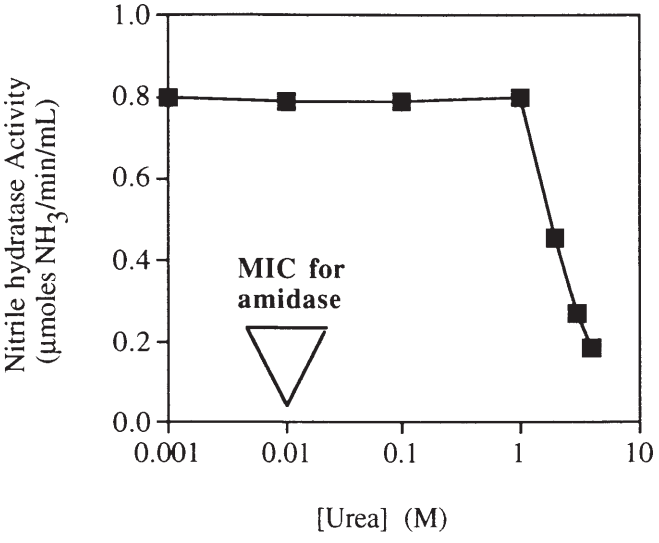

Fig. 10. Effect of urea on *Bacillus* RAPc8 nitrile hydratase and amidase activities

applied to industrial biocatalysis and biotransformation processes.

Acrylamide production using thermophilic nitrile hydratases

Acrylonitrile hydrolysis trials using *Bacillus* RAPc8 cells immobilized by encapsulation in sodium alginate (Graham, Barfield, Pereira, and Cowan, manuscript in preparation) indicated that the intracellular hydratase and amidase activities were remarkably resistant to inactivation (Fig. 9), even at acrylonitrile concentrations approaching those required for efficient industrial biocatalysis (0.75–1.0 M). In a nonoptimized batch reaction, up to 500 mM acrylonitrile substrate concentrations were efficiently catalyzed (greater than 60% conversion in 5 h). This *Bacillus* RAPc8-catalyzed whole-cell reaction converts acrylonitrile to acrylic acid via the amide, although high levels of intracellular amidase activity in this organism prevent accumulation of the amide intermediate (Pereira, unpublished results). Methods of effectively blocking amidase activity to produce the intermediate would obviously be of substantial value in the possible application of this organism. A screen of potential amidase inhibitors indicated that urea was particularly effective (MIC = 10 mM; 100% inhibition at <100 mM). The absence of any inhibitory effect of urea on nitrile hydratase activity at concentrations less than 1 M (Fig. 10) provides a substantial "operational window" in which the amide may be generated.

References

Amarant T, Vered Y, Bohak Z (1989) Substrates and inhibitors of the nitrile hydratase and amidase of *Corynebacterium nitrilophilus.* Biotechnol Appl Biochem 11:49–59

Asano Y, Fujishiro K, Tani Y, Yamada H (1982) Aliphatic nitrile hydratase from *Arthrobacter* sp. J1: Purification and characterisation. Agric Biol Chem 46:1165–1174

Bandyopadhyay AK, Nagasawa T, Asano Y, Fujishiro K, Tany Y, Yamada H (1986) Purification and characterization of benzonitrilase from *Arthrobacter* sp. strain J-1. Appl Environ Microbiol 51:302–306

Bauer R, Hirrlinger B, Layh N, Stolz A, Knackmuss HJ (1994) Enantioselective hydrolysis of racemic 2-phenylpropionitrile and other (R,S)-2-arylpropionitriles by a new bacterial isolate, *Agrobacterium tumefaciens* strain D3. Appl Microbiol Biotechnol 42:1–7

Bestwick LA, Gronning LM, James DC, Bones A, Rossiter JT (1993) Purification and characterisation of a nitrilase from *Brassica nupus.* Physiol Plant 89:611–816

Bonnet D, Artaud I, Moali C, Petre D, Mansuy D (1997) Highly efficient control of iron-containing nitrile hydratases by stoichiometric amounts of nitric oxide and light. FEBS Lett 409:216–220

Bult CJ, White O, Olsen GJ, Zhou LX, Fleischmann RD, Sutton GG, Blake JA, Fitzgerald LM, Clayton RA, Gocayne JD, Kerlavage AR, Dougherty BA, Tomb JF, Adams MD, Reich CI, Overbeek R, Kirkness EF, Weinstock KG, Merrick JM, Glodek A, Scott JL, Geoghagen NSM, Weidman JF, Fuhrmann JL, Nguyen D, Utterback TR, Kelley JM, Peterson JD, Sadow PW, Hanna MC, Cotton MD, Roberts KM, Hurst MA, Kaine BP, Borodovsky M, Klenk HP, Fraser CM, Smith HO, Woese CR, Venter JC (1996) Complete genome sequence of the methanogenic Archaeon, *Methanococcus jannaschii.* Science 273:1058–1073

Cramp R, Gilmour M, Cowan DA (1997) Novel thermophilic bacteria producing nitrile-degrading enzymes. Microbiology (NY) 143:2313–2320

Cramp R (1998) Novel nitrile degrading enzymes in thermophilic bacteria. PhD thesis, University of London, London, 283pp

Dufour E, Storer AC, Menard R (1995) Engineering nitrile hydratase activity into a cysteine protease by a single mutation. Biochemistry 34:16382–16388

Duran R, Nishiyama M, Horinouchi S, Beppu T (1993) Characterization of nitrile hydratase genes cloned by DNA screening from *Rhodococcus erythropolis.* Biosci Biotechnol Biochem 57:1323–1328

Endo T, Watanabe I (1989) Nitrile hydratase of *Rhodococcus* sp. N-774 – purification and amino acid sequences. FEBS Lett 243:61–64

Gilligan T, Yamada H, Nagasawa T (1993) Production of S-(+)-2-phenylpropionic acid from (R,S)-2-phenylpropionitrile by the combination of nitrile hydratase and stereoselective amidase in *Rhodococcus equi* TG328. Appl Microbiol Biotechnol 39:720–725

Goldlust A, Bohak Z (1989) Induction, purification and characterisation of nitrilase of *Fusarium oxysporum* f. sp. *melonis*. Biotechnol Appl Biochem 11:581–601

Harper DB (1977a) Fungal degradation of aromatic nitriles: enzymology of C-N cleavage by *Fusarium solani.* Biochem J 167:685–692

Harper DB (1977b) Microbial metabolism of aromatic nitriles: enzymology of C-N cleavage by *Nocardia* sp. NCIB 11216. Biochem J 165:309–319

Harper DB (1985) Characterisation of a nitrilase from *Nocardia* sp. NCIB 11215, using *p*-hydroxybenzonitrile as sole carbon source. Int J Biochem 17:677–683

Hashimoto Y, Nishiyama M, Horinouchi S, Beppu T (1994) Nitrile hydratase gene from *Rhodococcus* sp. N-774: requirement for its downstream region for efficient expression. Biosci Biotechnol Biochem 58:1859–1865

Honda J, Teratani Y, Kobayashi Y, Nagamune T, Sasabe H, Hirata A, Ambe F, Endo I (1992) Light-induced oxidation of iron atoms in a photosensitive nitrile hydratase. FEBS Lett 301:177–180

Huang WJ, Jia J, Cummings J, Nelson M, Schneider G, Lindqvist Y (1997) Crystal structure of nitrile hydratase reveals a novel iron centre in a novel fold. Structure (Lond) 5:691–699

Ikehata O, Nishiyama M, Horinouchi S, Beppu T (1989) Primary structure of nitrile hydratase deduced from the nucleotide sequence of a *Rhodococcus* species and its expression in *Escherichia coli.* Eur J Biochem 181:563–570

Klenk HP, Clayton RA, Tomb JF, White O, Nelson KE, Ketchum KA, Dodson RJ, Gwinn M, Hickey EK, Peterson JD, Richardson DL, Kerlavage AR, Graham DE, Kyrpides NC, Fleischmann RD, Quackenbush J, Lee NH, Sutton GG, Gill S, Kirkness EF, Dougherty BA, Mckenney A, Adams MD, Loftus B, Peterson S, Reich CI, McNeil LK, Badger JH, Glodek A, Zhou LX, Overbeek R, Gocayne JD, Weidman JF, McDonald L, Utterback T, Cotton MD, Spriggs T, Artiach P, Kaine BP, Sykes SM, Sadow PW, Dandrea KP, Bowman C, Fujii C, Garland SA, Mason TM, Olsen GJ, Fraser CM, Smith HO, Woese CR, Venter JC (1997) The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus.* Nature (Lond) 390:364

Kobayashi M, Izui H, Nagasawa T, Yamada H (1993) Nitrilase in biosynthesis of the plant hormone indole-3-acetic-acid from indole-3-acetonitrile – cloning of the *Alcaligenes* gene and site-directed mutagenesis of cysteine residues. Proc Natl Acad Sci USA 90:247–251

Kobayashi M, Nagasawa T, Yamada H (1989) Nitrilase of *Rhodococcus rhodochrous* J1: purification and characterization. Eur J Biochem 182:349–356

Kobayashi M, Nishiyama M, Nagasawa T, Horinouchi S, Beppu T, Yamada H (1991) Cloning, nucleotide-sequence and expression in *Escherichia coli* of 2 cobalt-containing nitrile hydratase genes from *Rhodococcus rhodochrous* J1. Biochim Biophys Acta 1129:23–33

Kobayashi M, Komeda H, Yanaka N, Nagasawa T, Yamada H (1992a) Nitrilase from *Rhodococcus rhodochrous* J1: sequencing and overexpression of the gene and identification of an essential cysteine residue. J Biol Chem 267:20746–20751

Kobayashi M, Yanaka N, Nagasawa T, Yamada H (1992b) Primary structure of an aliphatic nitrile-degrading enzyme, aliphatic nitrilase, from *Rhodococcus rhodochrous* K22 and expression of its gene and identification of its active-site residue. Biochem 31:9000–9007

Komeda H, Kobayashi M, Shimizu S (1996a) Transcriptional regulation of the *Rhodococcus rhodochrous* J1 NitA gene encoding a nitrilase. Proc Natl Acad Sci USA 93:10572–10577

Komeda H, Kobayashi M, Shimizu S (1996b) Characterization of the gene cluster of high molecular mass nitrile hydratase (H-NHase) induced by its reaction-product in *Rhodococcus rhodochrous* J1. Proc Natl Acad Sci USA 93:4267–4272

Komeda H, Kobayashi M, Shimizu S (1996c) A novel gene-cluster including the *Rhodococcus rhodochrous* J1 NHlbA genes encoding a low molecular mass nitrile hydratase (L-NHase) induced by its reaction product. J Biol Chem 271:15796–15802

Kopf MA, Bonnet D, Artaud I, Petre D, Mansuy D (1996) Key role of alkanoic acids on the spectral properties, activity, and active-site stability of iron-containing nitrile hydratase from *Brevibacterium* R312. Eur J Biochem 240:239–244

Levy-Schil S, Soubrier F, Crutz-Le Coq A-M, Faucher D, Crouzet J, Petre D (1995) Aliphatic nitrilase from a soil-isolated *Comomonas testosteroni* sp.: gene cloning and overexpression, purification and primary structure. Gene 161:15–20

Li WZ, Zhang YQ, Yang HF (1992) Formation and purification of nitrile hydratase from *Corynebacterium pseudodiphteriticum* ZBB-41. Appl Biochem Biotechnol 36:171–181

Martinkova L, Olsovsky P, Prepechalova I, Kren V (1995) Biotransformations of aromatic dinitriles using *Rhodococcus equi* cells. Biotechnol Lett 17:1219–1222

Martinkova L, Stolz A, Knackmuss HJ (1996) Enantioselectivity of the nitrile hydratase from *Rhodococcus equi* A4 towards substituted (R,S)-2-arylpropionitriles. Biotechnol Lett 18:1073–1076

Mathew CD, Nagasawa T, Kobayashi M, Yamada H (1988) Nitrilase-catalysed production of nicotinic acid from 3-cyanopyridine in Rhodococcus rhodochrous J1. Appl Environ Microbiol 54:1030–1032

Meth-Cohn O, Wang MX (1997a) An in-depth study of the biotransformation of nitriles into amides and/or acids using *Rhodococcus rhodochrous* AJ270. J Chem Soc Perkin Trans 1:1099–1104

Meth-Cohn O, Wang MX (1997b) Rationalisation of the regioselective hydrolysis of aliphatic dinitriles with *Rhodococcus rhodochrous* AJ270. Chem Commun 1041–1042

Milborrow BV (1963) Aldocine dehydratase activity in *Pseudomonas* cell extracts. Biochem J 87:255–258

Millais AJ, Casson D, Lovelady J, Nicholson AM, Knowles CJ (1997) The biotransformation of *t*-butylacetonitrile and its boron-containing analogue trimethylamine cyanoborane by *Brevibacterium* R312. Arch Microbiol 168:164–168

Moreau JL, Bigey F, Azza S, Arnaud A, Galzy P (1994) The enzymatic production of adipic acid. Biocatalysis 10:325–340

Nagamune T, Kurata H, Hirata M, Honda J, Hirata A, Endo I (1990) Photosensitive phenomena of nitrile hydratase of *Rhodococcus* sp. N-771. Photochem Photobiol 51:87–90

Nagasawa T, Yamada H (1987) Nitrile hydratase is a quinoprotein – a possible new function of pyrroloquinoline quinone – activation of H_2O in an enzymatic hydration reaction. Biochem Biophys Res Commun 147:701–709

Nagasawa T, Mauger J, Yamada H (1990) A novel nitrilase, arylacetonitrilase, of *Alcaligenes faecalis* JM3 – purification and characterization. Eur J Biochem 194:765–772

Nagasawa T, Nanba H, Ryuno K, Takeuchi K, Yamada H (1987) Nitrile hydratase of *Pseudomonas chlororaphis* B23 – purification and characterization. Eur J Biochem 162:691–698

Nagasawa T, Ryuno K, Yamada H (1986) Nitrile hydratase of *Brevibacterium* R312 – purification and characterization. Biochem Biophys Res Commun 139:1305–1312

Nagasawa T, Shimizu H, Yamada H (1993) The superiority of the 3rd-generation catalyst, *Rhodococcus rhodochrous* J1 nitrile hydratase, for industrial production of acrylamide. Appl Microbiol Biotechnol 40:189–195

Nagasawa T, Takeuchi K, Nardidei V, Mihara Y, Yamada H (1991a) Optimum culture conditions for the production of cobalt-containing nitrile hydratase by *Rhodococcus rhodochrous* J1. Appl Microbiol Biotechnol 34:783–788

Nagasawa T, Takeuchi K, Yamada H (1991b) Characterization of a new cobalt-containing nitrile hydratase purified from urea-induced cells of *Rhodococcus rhodochrous* J1. Eur J Biochem 196:581–589

Nishiyama M, Horinouchi S, Kobayashi M, Nagasawa T, Yamada H, Beppu T (1991) Cloning and characterization of genes responsible for metabolism of nitrile compounds from *Pseudomonas chlororaphis* B23. J Bacteriol 173:2465–2472

Payne MS, Wu SJ, Fallon RD, Tudor G, Stieglitz B, Turner IM, Nelson MJ (1997) A stereoselective cobalt-containing nitrile hydratase. Biochemistry 36:5447–5454

Sensen CW, Klenk HP, Singh RK, Allard G, Chan CCY, Liu QY, Penny SL, Young F, Schenk ME, Gaasterland T, Doolittle WF, Ragan MA, Charlebois RL (1996) Organizational characteristics and information-content of an archaeal genome – 156 kb of sequence from *Sulfolobus solfataricus* P2. Mol Microbiol 22:175–191

Smith DR, Doucette Stamm LA, Deloughery C, Lee HM, Dubois J, Aldredge T, Bashirzadeh R, Blakely D, Cook R, Gilbert K, Harrison D, Hoang L, Keagle P, Lumm W, Pothier B, Qiu DY, Spadafora R, Vicaire R, Wang Y, Wierzbowski J, Gibson R, Jiwani N, Caruso A, Bush D, Safer H, Patwell D, Prabhaker S, McDougall S, Shimer G, Goyal A, Pietrokovski S, Church GM, Daniels CJ, Mao JI, Rice P, Nolling J, Reeve JN (1997) Complete genome sequence of *Methanobacterium thermoautotrophicum* delta H: functional analysis and comparative genomics. J Bacteriol 179:7135–7155

Spielvogel BF (1988) Pharmacologically active analogues of amino acids. Adv Boron Boranes 5:329–342

Stein JL, Marsh TL, Wu KY, Shizuya H, Delong EF (1996) Characterization of uncultivated prokaryotes – isolation and analysis of a 40 kilobase-pair genome fragment from a planktonic marine archaeon. J Bacteriol 178:591–599

Stevenson DE, Feng R, Duman F, Groleau D, Milhoc A, Storer AC (1992) Mechanistic and structural studies on *Rhodococcus* ATCC 39484 nitrilase. Biotechnol Appl Biochem 15:283–302

Sugai T, Yamakaki T, Yokoyama M, Ohta H (1997a) Biocatalysis in organic synthesis: the use of nitrile- and amide-hydrolyzing microorganisms. Biosci Biotechnol Biochem 61:1419–1427

Sugai T, Yokohama M, Yamazaki T, Ohta H (1997b) Privotal role of (E)-3-carbamoyl-1-propenylboronic acid in the combination of Suzuki-Miyaura coupling and enzyme reactions: synthesis of (3E,5E)- and (3E,5Z)-6-phenyl-3,5-hexadienoic acid. Chem Lett 8:797–798

Sugiura Y, Kuwahara J, Nagasawa T, Yamada H (1988) Significant interaction between low-spin iron (III) site and pyrroloquinoline quinone in active-center of nitrile hydratase. Biochem Biophys Res Commun 154:522–528

Takashima Y, Mukumoto F, Mitsuda S (1995) Production of amide compounds from nitrile compounds. Sumitomo-Chem, Osaka, Japan

Takashima Y, Mukumoto F, Mitsuda S (1996) Enhancement of hydrative activity of a thermophilic bacterium capable of producing an amide from the corresponding nitrile. Sumitomo-Chem, Osaka, Japan

Tani Y, Kurihara M, Nishise H (1989) Characterization of nitrile hydratase and amidase, which are responsible for the conversion of dinitriles to mononitriles, from *Corynebacterium* sp. Agric Biol Chem 53:3151–3158

Thimann KV, Mahadevan S (1964) Nitrilase it. Substrate specificity and possible made of action. Arch Biochem Biophys 107:62–68

Thimann KV, Mahadevan S (1964) Nitrilase: occurrence, preparation and general properties of the enzyme. Arch Biochem Biophys 105:133–141

Yamada H, Kobayashi M (1996) Nitrile hydratase and its application to industrial production of acrylamide. Biosci Biotechnol Biochem 60:1391–1400

Yamada H, Ryuno K, Nagasawa T, Enomoto K, Watanabe I (1986) Optimum culture conditions for production by *Pseudomonas chlororaphis* B23 of nitrile hydratase. Agric Biol Chem 50:2859–2865

Yamaki T, Oikawa T, Ito K, Nakamura T (1997) Cloning and sequencing of a nitrile hydratase gene from *Pseudonocardia thermophila* JCM3095. J Ferment Bioeng 83:474–477

Yamamoto K, Komatsu K (1991) Purification and characterization of nitrilase responsible for the enantioselective hydrolysis from *Acinetobacter* sp. AK226. Agric Biol Chem 55:1459–1466

Yamamoto K, Fujimatsu I, Komatsu YI (1992a) Purification and characterization of nitrilase from *Alcaligenes faecalis* ATCC 8750 responsible for enantioselective hydrolysis of mandelonitrile. J Ferment Bioeng 73:425–430

Yamamoto K, Ueno Y, Otsubo K, Yamane H, Komatsu KI, Tani Y (1992b) Efficient conversion of dinitrile to mononitrile-monocarboxylic acid by *Corynebacterium* sp. C5 cells during tranexamic acid synthesis. J Ferment Bioeng 73:125–129