The Utilization of Nitriles and Amides by a *Rhodococcus* Species

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A species of *Rhodococcus* was isolated from garden soil on the basis of its capacity to use acetonitrile as sole C and N source. Acetonitrile-grown cells hydrolysed a number of amides and nitriles to ammonia. The substrate nitriles, listed in order of decreasing hydrolysis rates, were acetonitrile, acrylonitrile, propionitrile and *n*-butyronitrile. The corresponding amides were also hydrolysed together with formamide and, to a small extent, nicotinamide. With the exception of acrylonitrile and acrylamide, each compound supported growth as did the non-substrates malonamide, benzamide, α -phenylacetamide and 3-aminopropionitrile. Benzonitrile, phenylacetoneitrile (benzyl cyanide), malononitrile and aminoacetonitrile did not support growth. Nicotinamide and benzamide stimulated acetamidase activity but malonamide had no effect. Both the aminonitriles inhibited the acetonitrilase system. Cells grown in succinate/(NH₄)₂SO₄ medium did not hydrolyse acetonitrile or acetamide indicating that the enzymes involved in nitrile degradation are subject to induction/repression. Acetamide and acetate appear to be gratuitous inducers of acetonitrilase: acetate also induces the acetamidase.

INTRODUCTION

Aliphatic mononitriles are commonly metabolized in bacteria by stepwise hydrolysis to the corresponding carboxylic acid and ammonia. Thus the pathway for acetonitrile breakdown is:

This reaction sequence was first suggested by Mimura *et al.* (1969) who found that ammonia and an amide, not specifically acetamide, accumulated in the medium when *Corynebacterium nitrilophilus* nov. sp. C-42 was grown on acetonitrile. Likewise, DiGeronimo & Antoine (1976) observed the sequential formation of acetamide and acetate by *Nocardia rhodochrous* LL100-21 under similar conditions. The first step in the pathway was confirmed by showing transfer of ¹⁴C from acetonitrile to acetamide in another nitrile degrader, a *Pseudomonas* sp. (group III; NCIB 10477) (Firmin & Gray, 1976). The pathway was shown in *Arthrobacter* sp. J-1 to involve two distinct enzymes, a nitrilase (or nitrile hydratase) and an amidase (Asano *et al.*, 1980).

Other nitriles which support bacterial growth when supplied as the sole source of C and/or N include a variety of unsubstituted aliphatic, alkyl and alkenyl mono- and di-nitriles (Mimura *et al.*, 1969; Fukuda *et al.*, 1971; DiGeronimo & Antoine, 1976; Yamada *et al.*, 1979, 1980), α -hydroxynitriles (Grant, 1973; Yamada *et al.*, 1979), α -aminonitriles (Fukuda

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et al., 1971) aromatic nitriles (Harper, 1977) and the natural product ricinine (Robinson & Hook, 1964).

Bacterial amidases have been most studied in species of *Pseudomonas* (Clarke, 1970, 1972) though these organisms are not known to utilize nitriles. *Pseudomonas aeruginosa* (PAC 1 wild-type) can only utilize acetamide and propionamide, whereas some strains of *P. putida* and *P. acidovorans* can also grow on *n*-butyramide (Clarke & Richmond, 1975).

Arnaud *et al.* (1976*a, b, c*) studied amide and nitrile hydrolysis by intact cells of a number of bacteria. However, almost nothing quantitative has been published about the specificities of the nitrilase and amidase systems of such nitrile degrading bacteria. The present work was designed to provide such information by measuring the relative rates of hydrolysis of 16 amides and nitriles by a newly isolated organism, a species of *Rhodococcus*. It was also intended to study the effect of non-substrate amides and nitriles on these reactions and to investigate the regulation of the enzymes involved.

METHODS

Isolation. The bacterium was obtained from soil by enrichment culture in basal salt medium (BSM) containing 0.1% (v/v) acetonitrile as sole C and N source. The purified isolate was identified as a *Rhodococcus* species by the NCIB.

Media. The BSM was that of Firmin & Gray (1976) except that $(\text{NH}_4)_2\text{SO}_4$ was normally omitted. It was sterilized by autoclaving at 103.5 kPa (15 lbf in⁻²) for 15 min, together with any added sodium acetate (1%, w/v), sodium succinate (1%, w/v) or $(\text{NH}_4)_2\text{SO}_4$ (0.1%, w/v). Nitriles and amides were introduced through sterile membrane filters (Oxoid, 0.45 μm) as a 5 to 10% (w/v or v/v) solution in BSM. Acrylonitrile, benzonitrile and phenylacetoneitrile could not be filter-sterilized. Aqueous acrylonitrile attacked the membranes while the other nitriles were insufficiently soluble. Therefore, these compounds were each transferred aseptically directly to the culture medium. None of them supported growth, so the result was unambiguous.

Growth studies. All cultures were grown at 25 °C in a linear shaker. Starter cultures (50 ml), inoculated from plates, were centrifuged aseptically at 12 100 *g* for 10 min and the cells resuspended to the same volume in sterile phosphate buffer (0.1 M-sodium phosphate, pH 7.0) to provide an inoculum (5 ml) for the test medium (95 ml). Samples were then removed aseptically at intervals and their A_{640} recorded.

To study the effect of acetonitrile concentration on yield, starter cultures were grown in BSM containing 0.1% (v/v) acetonitrile for 2 d and then transferred to fresh BSM containing various concentrations of acetonitrile.

The ability of various nitriles and amides to support growth was also investigated. Starter cultures were grown for at least 4 d usually in BSM containing $(\text{NH}_4)_2\text{SO}_4$ and sodium acetate. When succinate was to be used subsequently as an additional C source, acetate was replaced by sodium succinate. The test medium was BSM containing the relevant nitrile or amide (final concentration 0.5%, w/v or v/v). Where appropriate, an additional N source [$(\text{NH}_4)_2\text{SO}_4$] or an additional C source (acetate or succinate) was also introduced.

Growth of cultures used for enzyme assays. Starter cultures (5 × 50 ml) were grown in BSM plus 0.25% (v/v) acetonitrile for 3 d as before and then transferred to 4750 ml of the same medium in a 15 l aspirator. The culture was vigorously aerated with sterile filtered air supplied by a Hy-flo pump (Medcalf Bros, Potters Bar, U.K.). It was harvested using a Sharples continuous centrifuge (Pennwalt, Camberley, U.K.) at late-exponential phase (about 24 h growth), when total nitrilase and amidase activities were maximal: these activities decreased sharply during the stationary phase. The cells were resuspended to 100 ml in 0.1 M-sodium phosphate buffer (pH 7.0), giving a concentrated suspension containing typically 10 mg dry weight cells ml⁻¹.

Preparation of cell extracts. All stages were carried out at 0 to 4 °C. Cell extracts were prepared by sonicating 50 ml of the concentrated cell suspension for a total of 30 min using a 150 W Dawe Soniprobe (Dawe Instruments, London, U.K.). The probe was cooled in ice/water for 30 s and then used to sonicate the suspension for 1 min; the cycle was then repeated. The sonicated suspension was centrifuged at 17 400 *g* for 15 min and the supernatant fraction retained.

Estimation of nitrilase and amidase activities. Rates of ammonia liberation from the test nitriles and amides were determined. Intact cells released this ammonia into the medium so the enzymes could be assayed *in situ*. The nitrilase assay made use of the natural coupling between nitrilase and the endogenous amidase.

The assay was done at 25 °C and all components were pre-equilibrated to this temperature. Bacterial suspensions were diluted with an equal volume of 0.1 M-sodium phosphate buffer (pH 7.0) containing sufficient nitrile or amide to give a final concentration of 0.05 M. The reaction mixture was normally incubated until 2 to 6% of the substrate had been hydrolysed to ammonia. If hydrolysis was rapid, the cell suspension was further diluted with buffer. If hydrolysis was slow, the incubation period was extended to 30 min for amides and 120 min for nitriles.

The reaction was terminated by mixing 0.5 ml samples with 0.5 ml 0.02 M aqueous AgNO_3 in a microdiffusion vessel, and then 1.0 ml saturated aqueous K_2CO_3 was added. The K_2CO_3 would not, by itself, terminate the reaction effectively. Microdiffusion was carried out by a modification of the technique described by Etherington & Morrey (1967) for 2 h at room temperature with continuous agitation. Ammonia recovered in the acid trap was measured using Nessler's reagent (BDH diluted 20-fold with deionized distilled water) and the absorbance at 420 nm recorded. Standard $(\text{NH}_4)_2\text{SO}_4$ samples were included in each set of determinations. Results were corrected for chemical decomposition of the substrates, which was substantial in the case of formamide.

Transferase activity was estimated by the method of Brammar & Clarke (1964) except that 0.1 M-sodium phosphate buffer, pH 7, was used and the incubation temperature was 25 °C.

The hydrolase and transferase activities were related to cell dry weights, measured by washing concentrated bacterial suspensions once with water and drying 1.0 ml samples at 105 °C for 2 h.

Attempts to detect aminonitrile hydrolysis by NMR. Acetonitrile-grown cells were incubated with either aminoacetonitrile hydrochloride or 3-aminopropionitrile fumarate as for the usual enzyme assays, except that the incubation period was 5 h. These suspensions (5 ml) were centrifuged at 2500 g for 10 min and the supernatant fractions evaporated to dryness *in vacuo* at 25 °C. The residues were taken up in 0.5 ml D_2O and examined with a 100 MHz NMR spectrometer (Varian Associates, Palo Alto, U.S.A.). The spectrometer was capable of distinguishing between nitriles and their corresponding amides, at least for acetonitrile and aminoacetonitrile.

Enzyme induction studies. The *Rhodococcus* sp. was grown in 5 l batch culture as before except that acetonitrile was replaced by alternative C and N sources: starter and test media were normally identical. If acetamidase activity was not detectable, acetonitrile hydrolysis was assayed indirectly by measuring the rate of ammonia production in the presence of *Pseudomonas putida* PPE1 (A87). This organism was grown in an acetamide-containing medium (Kelly & Clarke, 1962) to induce aliphatic amidase activity (Clarke, 1972) but no nitrilase activity was detected. The reaction mixture, containing 2 ml amidase-negative *Rhodococcus* cells, 1 ml *P. putida* cells (these contained about 40 nkat amidase activity) and 1 ml 0.2 M-acetonitrile in phosphate buffer, was incubated at 25 °C for 30 min. Ammonia concentrations were determined directly (Burris, 1972) by mixing 2 ml of sample with 2 ml Nessler's reagent and 3 ml 2 M-NaOH: the A_{490} was recorded 5 min later.

RESULTS

Growth studies

The growth yield was approximately proportional to acetonitrile concentration between 0.05 and 0.25 % (v/v) (Fig. 1). At 0.5 % (v/v) there was a marked reduction in molar yield, while at 3 % (v/v) growth was virtually inhibited.

Table 1 shows that of the ten amides tested only one, acrylamide, failed to support growth of the *Rhodococcus* sp. when supplied as either the sole source of C or N. The corresponding

Fig. 1. Effect of acetonitrile concentration on the growth of the *Rhodococcus* sp. in minimal medium.

nitrile, acrylonitrile, was also unable to support growth. The bacterium could not, however, grow with every nitrile whose corresponding amide was utilized, e.g. benzonitrile, phenylacetoneitrile and malononitrile. 3-Aminopropionitrile was a N source, but could not easily be tested as a C source since it was supplied as the fumarate salt.

Hydrolysis of nitriles and amides by acetonitrile-grown cells

When the reaction was allowed to go to completion, acetonitrile and acetamide gave 80% and 100%, respectively, of the theoretical ammonia yields.

Of the amides, only acetamide, propionamide and acrylamide were rapidly hydrolysed, followed by formamide, *n*-butyramide and nicotinamide (Table 2). Cell extracts having substantial acetamidase activity, like intact cells, were apparently unable to release ammonia from benzamide, α -phenylacetamide and malonamide even when the incubation period was extended to 120 min. Thus the negative results obtained with intact cells were not simply due to permeability barriers.

The relative rates of hydrolysis of the nitriles did not parallel those of the corresponding amides. The rate of nitrile hydrolysis to yield ammonia decreased the greater the number of C-atoms per molecule. Moreover, the introduction of a double bond, as in the $\text{CH}_3\cdot\text{CH}_2$ to $\text{CH}_2\text{:CH}$ conversion, stimulated the amidase but depressed the nitrilase.

Table 1. *Growth of the Rhodococcus sp. on various nitriles and amides*

Growth was positive if the A_{640} of the culture increased to >1.00 within the 7 d incubation period. The non-growing cultures did not show any significant increase in A_{640} during the same period.

Compounds supporting growth when supplied as sole C and N source	Compounds supporting growth when supplied as sole N source	Compounds not supporting growth when supplied as sole C or N source
Acetonitrile	3-Aminopropionitrile (+ fumarate)	Acrylonitrile
Acetamide	Formamide (+ acetate or succinate)	Acrylamide
Propionitrile	Malonamide (+ acetate or succinate)	Malononitrile
Propionamide	Nicotinamide (+ acetate or succinate)	Aminoacetonitrile (bisulphate)
<i>n</i> -Butyronitrile	Benzamide (+ acetate)	Benzonitrile
<i>n</i> -Butyramide	Urea (+ acetate)	Phenylacetoneitrile
α -Phenylacetamide		

Table 2. *Hydrolysis of nitriles and amides by acetonitrile-grown cells of Rhodococcus sp.*

Results are the averages of duplicate measurements. The value for HCN is scarcely greater than experimental uncertainty. Malononitrile, benzonitrile and phenylacetoneitrile were not tested: the corresponding amides were not hydrolysed so the coupled assay would not have been valid. The hydrolysis rates are expressed as nmol NH_3 produced min^{-1} (mg dry wt cells) $^{-1}$.

Nitrile	Hydrolysis rate	Corresponding amide	Hydrolysis rate
Hydrogen cyanide	0.3	Formamide	360 ± 40
Acetonitrile	86 ± 3	Acetamide	2100 ± 200
Propionitrile	2.1 ± 0.1	Propionamide	9100 ± 1200
<i>n</i> -Butyronitrile	0.87 ± 0.01	<i>n</i> -Butyramide	65 ± 6
Acrylonitrile	27 ± 4	Acrylamide	4500 ± 400
Aminoacetonitrile	ND	—	—
3-Aminopropionitrile	ND	—	—
		Nicotinamide	1.6 ± 0.4
		Benzamide	ND
		α -Phenylacetamide	ND
		Malonamide	ND

ND, Not detected.

Table 3. *Nitrilase and amidase activities in cells grown on different C and N sources*

Acetonitrile was added to 0.25% (v/v) and acetamide to 0.25% (w/v). The concentrations of the other C and N sources are given in Methods. The starter medium for the last experiment contained no acetamide to avoid initial induction of the enzymes. Results are the averages of duplicate assays.

C/N source(s) in growth medium	Enzyme activity [nmol NH ₃ produced min ⁻¹ (mg dry wt cells) ⁻¹]	
	Acetonitrilase	Acetamidase
Acetonitrile	86 ± 3	2100 ± 200
Acetamide	113 ± 4	1240 ± 40
Sodium acetate + (NH ₄) ₂ SO ₄	17.9 ± 0.5	520 ± 20
Sodium succinate + (NH ₄) ₂ SO ₄	ND	ND
Sodium succinate + Acetamide + (NH ₄) ₂ SO ₄	25.1 ± 0.7	363 ± 4

ND, Not detected.

The hydrolysis of the aminonitriles to the corresponding amino acids and ammonia could fail to occur at either the nitrile or the amide level. Thus an NMR spectrometer was used to search for aminoamides in the relevant reaction mixtures. None were detected. Standards were used to determine the sensitivity of the spectrometer. These measurements suggested that the rates of hydrolysis of aminoacetonitrile and 3-aminopropionitrile to their corresponding amides cannot be greater than 1.7 and 3.4, respectively, using the same units as in Table 2.

Several bacteria have been shown to transfer the acyl moiety of amides to hydroxylamine as well as to water (Clarke, 1970): this activity was also detected with the *Rhodococcus* sp. With acetamide as substrate the transferase activity was approximately ninefold greater than the equivalent amide hydrolase activity, i.e. $19\,600 \pm 30$ nmol acethydroxamate produced min⁻¹ (mg dry wt of cells)⁻¹.

Effect of various compounds on enzyme activity

Compounds unable to act as effective substrates were tested as possible inhibitors or activators of nitrilase and amidase activities. Acetonitrile-grown cells were used for these experiments and the potential modifiers were supplied at the same concentrations as the substrates (0.05 M).

With acetamide as substrate, nicotinamide and benzamide stimulated NH₃ production by 19% and 18%, respectively, in whole bacteria and by 44% and 14% in cell extracts. Malonamide had no significant effect on acetamidase activity. With acetonitrile as substrate, and bacterial suspensions which had been sonicated for 5 min so that 5 to 10% of the cells had been broken, aminoacetonitrile and 3-aminopropionitrile decreased NH₃ formation by 85% and 30%, respectively.

Enzyme induction/repression studies

Nitrilase and amidase activities were present in bacteria grown in minimal media containing acetonitrile, acetamide or acetate and (NH₄)₂SO₄ (Table 3). When acetamide was present, succinate and/or (NH₄)₂SO₄ reduced formation of these enzymes: when acetamide was omitted suppression was complete.

DISCUSSION

The *Rhodococcus* sp. used here is moderately tolerant of high acetonitrile concentrations and is similar to *Nocardia rhodochrous* LL100-21 in that growth is virtually inhibited at 3%

(v/v) acetonitrile (DiGeronimo, 1975). *Corynebacterium nitrilophilus* nov. sp. C-42 will tolerate up to 5% (v/v) (Mimura *et al.*, 1969), while the *Pseudomonas* species isolated by Firmin & Gray (1976) will not grow even in 1% (v/v) acetonitrile.

Bacterial amidases are commonly studied in intact cells by measuring the ammonia released into the medium. Here this approach has been extended to study the relative rates of nitrile hydrolysis in a species of *Rhodococcus*. Fortunately even in the most unfavourable case, the amide is hydrolysed 24 times faster than the nitrile, so the nitrilase reaction will be rate-limiting. These hydrolysis reactions are almost quantitative, especially in dilute bacterial suspensions like those used for the amidase assay. Thus they provide a convenient way of investigating the specificities of the relevant enzymes *in vivo* together with those of any linked transport systems.

The coupled assay itself has established that aminoacetonitrile is not a substrate for *Rhodococcus* nitrilase: it is now known that the conversion of glycynamide to ammonia occurs spontaneously and quantitatively during microdiffusion. The situation regarding 3-aminopropionitrile is less clear, though NMR measurements show that it was not a good nitrilase substrate. Thus, aminoacetonitrile itself must be an active inhibitor while 3-aminopropionitrile might have been hydrolysed to the corresponding amide before it was effective. Since these inhibitions were demonstrated in a predominantly whole cell system, they could be due to competition between the aminonitriles and acetonitrile for a permease.

The amidase induced in acetonitrile-grown cells could hydrolyse acrylamide rapidly although this compound could not support growth. Acrylamide may not itself be an amidase inducer, but it was tested as a N source in the presence of the proven inducer, acetate. Thus either acrylamide or its hydrolysis product, acrylic acid, is toxic or else acrylamide acts as a repressor of amidase synthesis in this *Rhodococcus* sp. Similar arguments apply to the results obtained for acrylonitrile.

Malonamide, benzamide and α -phenylacetamide provide examples of the opposite situation: they are not substrates of the amidase present in acetonitrile-grown cells, but support growth. This suggests that the bacterium can synthesize more than one amidase. Although no ammonia was released from 3-aminopropionitrile, the growth-supporting properties of this nitrile are less certainly due to the induction of a new enzyme: NMR spectra show that 3-aminopropionitrile preparations include a one-carbon compound whose concentration increases with time. Thus, the N source in these growth experiments could be a chemical decomposition product.

The comparative rates of hydrolysis of the substrate amides by the *Rhodococcus* sp. are very similar to those of induced cells of *P. aeruginosa* (Kelly & Clarke, 1962). This is an interesting observation considering the diverse taxonomic positions of the two bacteria.

Rhodococcus, like *C. nitrilophilus* nov. sp. C-42 (Mimura *et al.*, 1969), *N. rhodochrous* LL100-21 (DiGeronimo & Antoine, 1976) and *Arthrobacter* sp. I-9 (Yamada *et al.*, 1979) cannot utilize benzonitrile. However, *N. rhodochrous* (NCIB 11216) (Harper, 1977) can metabolize the compound, probably by hydrolysing it directly to benzoic acid. Here the amide appears not to act as an intermediate since it could not readily support growth or act as a substrate for benzonitrile-grown cells. This breakdown pathway is similar to that of ricinine in bacteria (Robinson & Hook, 1964) and of indole-3-acetonitrile in higher plants (Thimann & Mahadevan, 1958).

During some of the present induction/repression studies *P. putida* was used to provide added amidase. This technique requires that any acetamide produced by the *Rhodococcus* sp. should be released into the medium. This is a reasonable assumption since *N. rhodochrous* LL100-21 and *C. nitrilophilus* nov. sp. C-42 both release amide into the culture filtrate when growing in the presence of acetonitrile (DiGeronimo & Antoine, 1976; Mimura *et al.*, 1969).

Both the amidase and nitrilase of *Rhodococcus* are clearly subject to induction. DiGeronimo & Antoine (1976) also found that the hydrolysis of acetonitrile by *N. rhodochrous* LL100-21 required prior induction, whereas the hydrolysis of α -aminonitriles by

Corynebacterium sp. HR3 to the corresponding amino acids was shown to be constitutive (Fukuda *et al.*, 1971).

The results in Table 3 suggest that in *Rhodococcus* the nitrilase and amidase are induced and repressed by the same factors though not to the same degree. Succinate, or one of its metabolites, probably represses both enzymes, an example of the widespread phenomenon of catabolite repression. Acetonitrile-grown cells contain both enzymes, though it is uncertain whether the nitrile or its hydrolysis product, acetamide, is the effective amidase inducer. The gratuitous induction of the nitrilase by acetamide and of both enzymes by acetate also occurs in *Brevibacterium* R312 (Arnaud *et al.*, 1977; Jallageas *et al.*, 1978). The aliphatic amidase of *P. aeruginosa* is also induced when grown in acetate medium (Clarke, 1970).

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