Nitrile hydrolysis by *Rhodococcus erythropolis* BL1, an acetonitrile-tolerant strain isolated from a marine sediment

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A number of physiologically different nitrile-hydrolysing bacteria were isolated from coastal marine sediments in Denmark by enrichment culture. One strain, BL1, identified as *Rhodococcus erythropolis*, grew on acetonitrile as sole carbon and nitrogen source in a defined medium. Growth occurred between 0 and 8% NaCl with an optimum around 2%, thus reflecting the marine origin of the isolate. Intact cells of *R. erythropolis* BL1 could hydrolyse a large variety of saturated and unsaturated aliphatic nitriles to their corresponding acids. Benzonitrile and benzylocyanide were not hydrolysed, whereas some aromatic compounds containing a -CN group attached to a C₃ or C₄ aliphatic side chain were accepted as substrates. The substrate spectrum of *R. erythropolis* BL1 was thus markedly different from those of other Gram-positive nitrile-hydrolysing bacteria isolated from non-marine environments. Nitrile hydrolysis during growth and in resting cell suspensions usually occurred without intermediate accumulation of amide outside the cells. Detailed studies, however, showed that nitrile hydrolysis by strain BL1 was due to a nitrile hydratase/amidase enzyme system. Nitrile hydratase activity was found to be inducible whereas amidase activity was constitutive. The amidase activity of cells could, however, be enhanced manyfold by growth in media containing acetamide or acetonitrile. In most cases amides were hydrolysed at a much higher rate than the corresponding nitriles, which explained why amides were rarely detected in the surrounding medium during nitrile hydrolysis. *R. erythropolis* BL1 exhibited the highest tolerance towards acetonitrile ever reported for a nitrile-hydrolysing bacterium, as demonstrated by its ability to grow exponentially in the presence of 900 mM acetonitrile.

Keywords: *Rhodococcus erythropolis*, nitrile hydratase, amidase, marine sediments, substrate tolerance

INTRODUCTION

Several micro-organisms have been reported to hydrolyse both aliphatic and aromatic nitriles (DiGeronimo & Antoine, 1976; Harper, 1977; Jallageas *et al.*, 1980; Asano *et al.*, 1982; Ingvorsen *et al.*, 1988; Nawaz *et al.*, 1992). In bacteria, hydration of nitriles proceeds by two different enzymic pathways: (1) a hydratase/amidase enzyme system which catalyses the reaction to the corresponding carboxylic acid and ammonia in a two-step reaction via a free amide intermediate, or (2) a nitrilase enzyme which catalyses the reaction in one step. In addition to these

hydration reactions, reduction of nitriles by nitrogenase enzymes has been described (Jallageas *et al.*, 1980; Finnegan *et al.*, 1992).

Many naturally occurring nitriles have been described (Jallageas *et al.*, 1980; Vennesland *et al.*, 1981). However, only a few (e.g. ricinine and mandelonitrile) have been reported to be hydrolysed by bacteria (Robinson & Hook, 1964; Yamamoto *et al.*, 1991). Indeed, most nitrile-hydrolysing bacteria described in detail have been enriched and finally isolated using media containing synthetic nitriles as carbon and/or nitrogen sources.

Many synthetic nitrile compounds are used in industrial operations (Wyatt & Linton, 1988). The widespread usage of nitriles has created considerable commercial

Abbreviations: AHA, amide-hydrolysing activity; NHA, nitrile-hydrolysing activity.

interest in nitrile-hydrating enzymes, both as biocatalysts for chemical syntheses (Nagasawa & Yamada, 1989) and as a means of biological detoxification of nitrile-containing waste (Knowles & Wyatt, 1992; Chapatwala *et al.*, 1993). Industrial usage of nitriles may result in discharge of synthetic nitriles into marine, freshwater and soil environments, where they may be detoxified by natural populations of nitrile-hydrolysing micro-organisms. All nitrile-hydrolysing bacteria described in the literature appear to have been isolated from soils or freshwater environments (DiGeronimo & Antoine, 1976; Harper, 1977; Jallageas *et al.*, 1980). The present study was initiated with the aim of gaining information about nitrile-hydrolysing bacteria in marine sediments. We report here the isolation and characterization of a marine strain capable of hydrolysing organic nitriles.

METHODS

Composition of media. The defined basal medium (DE medium) used for enrichment and isolation of acetonitrile-utilizing micro-organisms contained the following components (g per litre Milli-Q Plus water): NaCl, 20; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 3; KCl, 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.15; K_2HPO_4 , 1.03; KH_2PO_4 , 0.75; and 1 ml each of the following three solutions: trace element solution (TES-3), vitamin mixture (VM) and vitamin B_{12} solution. TES-3 contained (in mg per litre Milli-Q Plus water): $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 2000; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 250; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 100; ZnCl_2 , 70; H_3BO_3 , 6; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 40; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 70; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 2; $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, 60; $\text{NaWO}_4 \cdot 2\text{H}_2\text{O}$, 6. The VM contained the following seven vitamins (in mg per litre 25 mM sodium phosphate buffer, pH 7.0): pyridoxamine dihydrochloride, 100; calcium D(+)-pantothenate, 50; nicotinic acid, 100; DL- α -lipoic acid, 10; folic acid, 30; D-(+)-biotin, 10; 4-aminobenzoic acid, 40. The vitamin B_{12} solution contained 50 mg cyanocobalamin per litre Milli-Q Plus water. The pH of the DE medium was adjusted to 7.2 with NaOH or H_3PO_4 before autoclaving. After heat sterilization (121 °C, 20 min) and cooling, one ml of a 0.2 μm filter-sterilized solution of thiamin hydrochloride (100 mg per litre 10 mM sodium phosphate buffer, pH 3.4) was added to the medium. Solid DE medium contained 15 g Bacto agar (Difco) l⁻¹. Agar plates of complex medium (CM) were used for testing the purity of nitrile-hydrolysing isolates. CM had the following composition (in g per litre DE medium): beef extract, 8; yeast extract, 1; Bacto agar, 15. Unless otherwise stated, all media, substrates and reagent solutions were prepared in Milli-Q Plus water.

Enrichment and isolation of bacteria. Samples of marine sediment were collected from different localities on the east and west coasts of Denmark. Enrichments were initiated by adding 1 ml sediment sample to 100 ml DE medium in 500 ml Erlenmeyer flasks with aluminium caps used for aerobic cultivation of micro-organisms (hereinafter referred to as 'experimental flasks'). Finally acetonitrile, serving as the sole carbon and nitrogen source, was added aseptically to the experimental flasks to a final volumetric concentration of 0.5% (95 mM) (hereafter referred to as DE/acetonitrile medium). Enrichment flasks were incubated aerobically at 23 °C on a gyrotory incubator (Innova, model 4000, New Brunswick Scientific) at 180 r.p.m., and checked daily for production of ammonia (as an indication of nitrile hydrolysis). If positive, 1 ml of the culture was transferred to a new experimental flask with DE/acetonitrile medium. After several transfers, cultures still showing ammonia production were diluted serially in DE

medium and plated on DE/acetonitrile agar medium. Well-separated colonies were picked and transferred to liquid DE medium and checked for nitrile-hydrolysing activity as described below. Nitrile-hydrolysing isolates were finally checked for purity by plating on nitrile-free CM. Unless otherwise stated, isolates were maintained and grown in 100 ml DE/acetonitrile medium at 23 °C on a gyrotory incubator at 180 r.p.m.

Identification of micro-organisms. Initial identifications of the isolates were carried out according to Goodfellow & Lechevalier (1989) and Goodfellow (1989). The biochemical tests and staining procedures were performed as described by Doetsch *et al.* (1981). Final identification of strain BL1 described in the present study, by chemotaxonomy and 16S rRNA analysis was done by DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany).

Preparation of washed cells. Cells grown to mid-exponential phase in DE/acetonitrile medium ($\text{OD}_{600} \sim 2-3$) were harvested by centrifugation at 6500 g in a Sorvall centrifuge (model RC-5, Dupont Instruments) for 10 min at 10 °C. The cells were washed twice in 100 mM phosphate buffer (pH 7.0) and resuspended in phosphate buffer. The washed cell suspension was used immediately or stored overnight at 4 °C.

To avoid carry-over of medium components, growth experiments were initiated by using an inoculum of washed cells prepared as described above, but under aseptic conditions (aseptically washed cells).

Salt range for growth. To study the effect of NaCl on growth, eight experimental flasks were prepared with DE medium containing the following additions of NaCl: 0, 1, 2, 3, 4, 5, 6 and 8% (w/v). The flasks were inoculated with a suspension of aseptically washed cells (pregrown at 2% NaCl) to an initial OD_{600} of ~ 0.2 . Acetonitrile (0.5%, v/v) was added as sole carbon and nitrogen source. Incubation was carried out under the conditions described above, and growth was evaluated by measuring OD_{600} after 120 h incubation.

Growth at different concentrations of acetonitrile. To investigate the kinetics of growth at different concentrations of acetonitrile, DE medium was supplemented with 30, 50, 300, 500 or 1000 mM acetonitrile as sole carbon and nitrogen source. The experimental flasks were inoculated with a suspension of aseptically washed cells to an initial OD_{600} of approximately 0.2, and incubated under the conditions described above. At intervals samples (2 ml) were aseptically removed from the cultures for measurement of OD_{600} and pH. Afterwards the samples were centrifuged and the cell-free supernatant withdrawn and stored at -20 °C until analysed for metabolites as described below.

Nitriles and amides as growth substrates. A number of nitriles and amides were tested for their ability to serve as sole carbon and nitrogen source for growth of strain BL1. Potential substrates were added to experimental flasks, which were inoculated with a suspension of aseptically washed cells pregrown in DE/acetonitrile medium. Liquid substrates were added to a final concentration of 0.3% (v/v) and solid substrates to a final concentration of 30 mM. Growth was evaluated as OD_{600} after 72 h incubation.

Assay for enzyme activities. Nitrile-hydrolysing activity (NHA) and amide-hydrolysing activity (AHA) were assayed by measuring the ammonia released from the nitrile and amide substrates, respectively, in reaction mixtures containing washed cells.

The amidase activity of strain BL1 was found to be substantially higher than the nitrile hydratase activity for most substrates.

Therefore, measurement of ammonia release from nitriles is a quantitative measurement of NHA in most instances. The reaction mixture contained 2 ml washed cells (diluted to a final OD₆₀₀ of 1–2) in 100 mM phosphate buffer (pH 7.0). The reaction was started by addition of the substrate to a final concentration of 30 mM. Incubation was at 30 °C in an Eppendorf thermomixer (model 5437). Samples (0.2 ml) of the reaction mixture were removed at 20 min intervals for 60 min and centrifuged for 5 min at 3500 *g* in a Sigma centrifuge (model 113). The cell-free supernatant was either analysed immediately for ammonia or stored (–20 °C) for subsequent analysis. Two types of controls were performed for each substrate tested to correct for ammonia released from cells and chemical hydration of substrates, respectively (Miller & Gray, 1982; Ingvorsen *et al.*, 1991). One unit of enzyme activity is defined as the amount of enzyme which produces 1 µmol NH₄⁺ min^{–1} under the conditions stated above.

Substrate specificity. The NHA and AHA assay procedures were used to test the enzyme specificities of washed cells of strain BL1 precultivated in DE/acetonitrile medium (0.5%, v/v) towards a wide range of nitriles and amides (see Table 1). In each case the concentration of substrate in the reaction mixture was 30 mM. Methanol (5%, v/v) was added to the reaction mixture to enhance the solubility of hydrophobic substrates. Control experiments with hydrophilic substrates showed that reaction rates were not affected by methanol.

Induction of NHA and AHA. The effects of various nitrogen compounds on NHA and AHA were tested after precultivation of strain BL1 for many generations in DE/acetonitrile medium (0.5%, v/v) or DE/glucose/ammonia medium (40 mM/15 mM), respectively. Cells precultivated in these two media (referred to as induced and non-induced cells, respectively) were harvested in the exponential growth phase, washed aseptically, and used to inoculate experimental flasks with DE medium supplemented with various carbon and nitrogen sources: acetonitrile (0.5%, v/v), acetonitrile/glucose (0.5%/40 mM), acetonitrile/glucose/ammonia (0.5%/40 mM/15 mM), glucose/ammonia (40 mM/15 mM), acetamide (50 mM), acetamide/glucose (50 mM/40 mM) and benzonitrile (20 mM). After 24 and 48 h growth in these test media, samples were withdrawn from each flask and assayed for NHA as described above. Some cultures were also assayed for amidase activity (AHA) after 24 h growth.

Analytical methods. Samples of cell suspensions from growth and activity experiments were withdrawn at intervals and the cells removed by centrifugation in a Sigma (model 113) centrifuge (5 min at 3500 *g*). The clear supernatant was removed and used for analyses of acetonitrile, acetamide, acetate and ammonia as described below. Acetonitrile and acetamide were determined by gas chromatography using a Chrompack model 438 A gas chromatograph equipped with a flame ionization detector connected to a Shimadzu CR 601 Chromatopack integrator. A stainless steel column (2 m × 2.4 mm i.d.) packed with Porapak Q (mesh 80–100) was used as analytical column. For acetonitrile the column, injector and detector temperatures were set at 175, 210 and 250 °C, respectively. For acetamide the temperatures were changed to 225, 250 and 300 °C, respectively. Nitrogen was used as carrier gas at a flow rate of 30 ml min^{–1}.

Acetate was analysed by reverse-phase chromatography using a Sykam high-performance liquid chromatograph (HPLC) system consisting of a pump (model S 1100), a column oven (model S 4110), a UV-detector (model LDS 3200) and an autosampler (Jasco, model 851-AS). The analytical column (300 mm × 7.8 mm; Aminex HPX-87H, Bio-Rad) was held at 60 °C. Elution was isocratic at a flow rate of 0.9 ml min^{–1} using

0.025 M H₂SO₄ as mobile phase. The absorbance was measured at 218 nm.

Ammonia was analysed by the salicylate/hypochlorite method according to Bower & Holm-Hansen (1980) using a Hitachi spectrophotometer (model U-2000).

Bacterial growth was followed by measurements of OD₆₀₀ with a Bausch and Lomb spectrophotometer (Spectronic 70). Samples were removed aseptically from the culture and if necessary diluted with Milli-Q Plus water to OD₆₀₀ values less than 1.0 prior to measurements.

Bacterial dry weight was determined by drying samples (2–10 ml) of culture broth previously washed with Milli-Q Plus water at 105 °C for 24 h. Protein was determined according to the procedure of Goa (1953) using BSA (Sigma) as standard. Calibration curves were constructed to relate OD₆₀₀ to dry weight (mg l^{–1}) and protein (mg l^{–1}). One unit of OD₆₀₀ corresponded to 615 mg dry weight l^{–1} or 313 mg protein l^{–1}.

Chemicals. All chemicals were of analytical-grade quality and purchased from commercial suppliers.

Replication of experiments. Growth experiments were done in duplicate; activity assays were done in triplicate and plotted as mean values.

RESULTS

Isolation of nitrile-hydrolysing bacteria and identification of isolate BL1

Five different nitrile-hydrolysing species were isolated from marine sediments sampled on the east and west coast of Denmark. A Gram-positive strain, designated BL1, obtained from Aarhus Bight sediment by repeated plating on DE/acetonitrile agar medium, was chosen for further studies due to its high NHA. The colonies of strain BL1 on DE/acetonitrile agar medium were 3–5 mm in diameter (4–6 d old), circular, convex with an entire margin, creamy and with a pink colour.

Cells of strain BL1 showed great morphological variability, from coccoid to rod-shaped with some tendency to form branching rods. The cells were Gram-positive, partially acid-fast and non-motile. Other physiological characteristics of the bacterium were: catalase positive, oxidase negative and non-spore-forming.

Cell wall analysis (fatty acid and mycolic acid) of strain BL1 resulted in its classification within the CMN taxon, which includes the families *Corynebacteriaceae*, *Mycobacteriaceae* and *Nocardiaceae*. A comparison of the 16S rRNA sequences showed strain BL1 to have 99.2% and 99.8% similarity, respectively, to *Rhodococcus erythropolis* and *Nocardia calcarica*. Due to recent reclassification, *N. calcarica* is now regarded as a synonym of *R. erythropolis* (Goodfellow, 1989; Rainey *et al.*, 1995), strain BL1 was therefore identified as *Rhodococcus erythropolis*.

Effect of NaCl on the growth of *R. erythropolis* BL1

R. erythropolis BL1 was capable of growth at salinities from 0 to 8% NaCl (Fig. 1). Cell yields (ΔOD₆₀₀ after 120 h incubation) were maximal between 1 and 3% NaCl. Incubations without added NaCl showed a cell yield

Fig. 1. Effect of salinity (NaCl concentration) on the growth of *R. erythropolis* BL1. Growth was evaluated as ΔOD_{600} after 120 h incubation.

Fig. 2. Growth of *R. erythropolis* BL1 on DE medium containing a low concentration of acetonitrile (30 mM). (a) $\circ$, Acetonitrile (sterile control); $\bullet$, acetonitrile; $\blacklozenge$, acetate; $\blacksquare$, ammonia. (b) $\triangle$, OD_{600} ; $\diamond$, pH; arrows indicate the start and end of the exponential growth phase.

corresponding to 80% of the maximum. At NaCl concentrations higher than 3%, cell yields decreased rapidly, but slow growth occurred at 8% NaCl.

Growth on and tolerance of acetonitrile

Strain BL1 was able to grow in DE medium with 30 mM acetonitrile as sole carbon and nitrogen source. A typical time course of cultivation is shown in Fig. 2. Upon inoculation, strain BL1 exhibited a lag phase of 12–15 h, during which small amounts of acetonitrile were hydrolysed to ammonia and acetate, which accumulated in the

Fig. 3. Growth of *R. erythropolis* BL1 on DE medium containing a high concentration of acetonitrile (1 M). (a) $\circ$, Acetonitrile (sterile control); $\bullet$, acetonitrile; $\blacktriangle$, acetamide; $\blacklozenge$, acetate; $\blacksquare$, ammonia. (b) $\triangle$, OD_{600} ; $\diamond$, pH; arrows indicate the start and end of the exponential growth phase.

medium. During exponential growth the acetonitrile concentration in the medium decreased rapidly and the ammonia concentration increased. Acetate produced from hydrolysis of acetonitrile served as carbon/energy source for growth and disappeared from the medium in mid-exponential growth (~ 25 h). Ammonia accumulated to a final concentration of 13 mM in the medium, causing the pH to increase from 7 to 8.

No acetamide was detected in the culture medium of strain BL1 during the experiment shown in Fig. 2. Also, acetamide was never detected extracellularly in resting cell suspensions hydrolysing acetonitrile (data not shown). The detection limit for acetamide was $\sim 100 \mu\text{M}$ by the GC method used. However, in rare instances, small amounts of amide were excreted by cells of strain BL1 (Fig. 3).

In order to elucidate the enzymic pathway of nitrile hydrolysis in strain BL1, inhibitor studies were performed using aldehydes as specific inhibitors of amidase activity (Amarant *et al.*, 1989). Acetaldehyde (2 mM) or formaldehyde (1 mM), when added to aseptically washed cells incubated with 30 mM acetonitrile, inhibited amidase activity of strain BL1, resulting in an accumulation of acetamide and a simultaneous cessation of ammonia production (data not shown). These inhibitor experiments strongly indicate the presence of a nitrile hydratase/amidase enzyme system in *R. erythropolis* BL1. The reason why amide intermediates were not usually detected outside cells of strain BL1 during nitrile hydrolysis could have been the presence of a high level of amidase activity.

This was confirmed in later experiments which showed that AHA was more than one order of magnitude higher than NHA (Table 1).

Cells of strain BL1 survived long exposures to high concentrations of acetonitrile. To investigate whether the strain was able to grow in the presence of high concentrations of acetonitrile, an experimental flask with DE medium containing 1 M acetonitrile was inoculated with an exponential-phase culture from DE medium supplemented with 0.5% (v/v) acetonitrile. A control flask containing sterile growth medium and 1 M acetonitrile was run in parallel in order to quantify evaporation of acetonitrile during incubation (Fig. 3). Inoculation of strain BL1 into DE medium containing 1 M acetonitrile resulted in a lag phase of about 50 h, i.e. about threefold longer than with 30 mM acetonitrile (Fig. 2). Little ammonia or acetate was excreted into the medium during the lag phase. The decline in acetonitrile concentration observed during this phase was mainly a result of evaporation (Fig. 3). Exponential growth started (~ 50 h) at an acetonitrile concentration of about 900 mM. Growth correlated with production of acetate and ammonia during the first half of the exponential growth period (45–90 h). Growth ceased at about 140 h as a result of pH inhibition. At this time ammonia production also ceased. Additional experiments showed that although strain BL1 tolerated exposure to pH 8.8 for many hours it did not grow above this pH value (data not shown). Low concentrations of acetamide (~ 0.5 mM) were detected in the culture medium during part of this experiment (Fig. 3), in contrast to the previous growth experiment (Fig. 2). When strain BL1 entered the stationary phase (~ 125 h) the culture medium contained 75 mM ammonia and 260 mM acetonitrile. A mass balance calculation showed that a total of about 340 mM of acetonitrile was hydrolysed by strain BL1. This value was estimated from the data in Fig. 3 assuming a total evaporation loss of approx. 370 mM acetonitrile (i.e. linear extrapolation between 0 and 130 h). Assuming an average C:N ratio of 5 for *R. erythropolis* BL1 cell synthesis (3.0 mg dry weight ml^{-1}) would require the assimilation of a maximum of 20 mM ammonia, thus leaving about 245 mM ammonia ($340 \text{ mM} - 20 \text{ mM} - 75 \text{ mM}$) unaccounted for. The low recovery of ammonia from the growth medium, which was also observed in the experiment of Fig. 2, is most likely due to ammonia stripping. Such a loss of ammonia was facilitated both by the continuous shaking employed and by the high pH (8.8) of the medium towards the end of growth (the dissociation constant pK_a for ammonia is 9.25). Additional experiments showed that significant amounts of ammonia disappeared from sterile experimental flasks containing ammonium chloride at pH 8.8.

The maximum growth rates measured at low (Fig. 2) and high (Fig. 3) acetonitrile concentrations were 0.15 h^{-1} and 0.07 h^{-1} , respectively, which indicated that acetonitrile had an inhibitory effect on the growth of strain BL1. However, this strain exhibited an exceptionally high tolerance to acetonitrile. The growth rate and the length of the lag phase did not differ significantly at acetonitrile concentrations below 300 mM. At 500 mM a much longer

Fig. 4. Rate of hydrolysis of acetonitrile by intact cells of *R. erythropolis* BL1 as a function of acetonitrile concentration. Hydrolysis was measured as the initial rate of ammonia release over 60 min. Insert: time course of ammonia production at 2 M acetonitrile.

lag phase was observed but the growth rate was identical (0.12 h^{-1}). Resting cells of *R. erythropolis* BL1 exhibited a rather high saturation concentration for hydrolysis of acetonitrile. Fig. 4 shows a plot of the initial hydrolytic velocity by intact cells of BL1 versus acetonitrile concentration. Maximum hydrolytic activity was measured at 500 mM, with more than 15% activity remaining at 2 M. The high maximum of acetonitrile hydrolysis obtained (Fig. 4) may reflect hydrolysis being limited by diffusion of substrate into the cells below 500 mM. A slow efflux of the intracellularly produced ammonia at lower nitrile concentrations could be another explanation. The sharp decline in hydrolytic activity observed above 500 mM acetonitrile is presumably due to substrate inhibition. It was, therefore, surprising that the hydrolytic activity was constant when washed cells were incubated for 6 h at 2 M acetonitrile, which was the highest concentration tested (cf. Fig. 4, insert).

Substrate specificity

The nitrile hydratase/amidase system of *R. erythropolis* BL1 exhibited broad substrate specificity. Only eleven of the thirty-seven nitriles tested were not hydrated by washed cell suspensions of *R. erythropolis* BL1 (Table 1). However, hydrolysis of some nitrile compounds tested may have escaped detection as substrates for the nitrile hydratase, since activity was evaluated by measuring ammonia production. The hydrolytic system of *R. erythropolis* BL1 had a clear preference for aliphatic saturated and unsaturated nitriles containing 2–4 carbon atoms, with propionitrile (C_3) being the most preferred substrate. The nitrile hydratase/amidase system of *R. erythropolis* BL1 also accepted side chains in the α -, β - and γ -positions (e.g. aminoacetonitrile, lactonitrile and 3-hydroxypropionitrile), although these substrates were hydrated at lower rates than their unbranched counterparts. Cyanide (CN^-/HCN) did not act as substrate (result not shown).

In contrast to *Rhodococcus rhodochrous* J1 and *Nocardia*

Table 1. Hydrolysis rates of various nitriles and amides by acetonitrile precultivated cells of *R. erythropolis* BL1

Rates of acetonitrile and acetamide hydrolysis corresponding to 47 U (g protein)⁻¹ and 1463 U (g protein)⁻¹, respectively, were taken as 100%. –, No hydrolysis.

Substrate	NHA (%)	Substrate	NHA (%)
Nitriles			
C₂		C₅	
Acetonitrile	100	2-Methyl 2-butenenitrile	126
Aminoacetonitrile	51	3-Pentenitrile	70
Chloroacetonitrile	47	Valeronitrile	47
Trichloroacetonitrile	42	Pivalinonitrile	28
C₃		C₆	
Propionitrile	2110	Iminodipropionitrile	49
Acrylonitrile	688	Adiponitrile	46
Methacrylonitrile	62	Mucononitrile	34
3-Hydroxypropionitrile	37	Aromatics	
Lactonitrile	37	Benzylpropionitrile	88
Malonitrile	–	Phenylacetonitrile	31
C₄		Cinnamonitrile	22
Butyronitrile	271	Benzonitrile	–
Allyl cyanide	174	Salicylonitrile	–
Succinonitrile	170	Phthalonitrile	–
4-Chlorobutyronitrile	143	4-Cyanopyrine	–
Isobutyronitrile	121	3-Cyanopyrine	–
Crotononitrile	22	Benzylcyanide	–
Fumaronitrile	6	Benzylaminoacetonitrile	–
Iminodiacetonitrile	–	Benzylmalonitrile	–
		Triphenylacetonitrile	–
Amides			
C₂		C₆	
Acetamide	100	Adipinamide	9
Chloroacetamide	25	Aromatics	
C₃		Phenylacrylamide	7
Propionamide	100	Isonicotinamide	6
Acrylamide	76	Salicylamide	6
Cyanoacetamide	13	Nicotinamide	3
N-Methylacetamide	8	Benzamide	3
Malonamide	–	Diphenylacetamide	–
C₄		Phthalamide	–
Butyramide	20		
Succinamide	9		

rhodochrous LL100-21 (Mauger *et al.*, 1988; Vaughan *et al.*, 1989) cells of strain BL1 were inactive towards a number of aromatic and heterocyclic substrates having a -CN group attached directly or closely to the ring structure (e.g. benzonitrile, benzylcyanide, 3-cyanopyridine) whereas -CN groups attached to a C₃ or C₄ aliphatic side chain were accepted as substrates (e.g. benzylpropionitrile, cinnamonitrile). Notably, cultivation of *R. erythropolis* BL1 in DE/acetonitrile medium containing benzo-

nitrile or benzylcyanide did not result in the induction of enzyme activities against these compounds.

The amidase of *R. erythropolis* BL1 exhibited highest activity towards short-chain aliphatic amides (i.e. acetamide and propionamide), the hydrolytic activity decreasing with increasing chain length or substitution (Table 1). Amide groups directly attached to the aromatic or heterocyclic ring (e.g. benzamide, nicotinamide) were

Table 2. Ability of nitriles and amides to support growth of *R. erythropolis* BL1

Growth was monitored after 72 h incubation. + + +, OD₆₀₀ > 3; + +, OD₆₀₀ 1–3; +, OD₆₀₀ < 1; –, no growth.

Substrate	Growth	Substrate	Growth
Acetonitrile	+ +	Acetamide	+ +
Propionitrile	+ + +	2-Chloroacetamide	+
Allyl cyanide	+ + +	Propionamide	+ + +
Butyronitrile	+ + +	N-Methylacetamide	+
Isobutyronitrile	+	Butyramide	+ + +
Fumaronitrile	+	Benzamide	+ + +
2-Methyl 2-butenenitrile	+		
Benzonitrile	–		

Table 3. Expression of NHA and AHA in *R. erythropolis* BL1 after growth in different types of defined media

Experimental flasks were inoculated with cells, which were either pregrown for several passages in (A) DE/glucose/ammonia medium or in (B) DE/acetonitrile medium. NHA and AHA are expressed as U (g protein)^{–1}. NHA of inoculum cultures: (A): 0 U (g protein)^{–1}, (B): 73 U (g protein)^{–1}. ND, Not done.

Growth medium	NHA (24 h)	NHA (48 h)	AHA (24 h)
(A) Non-induced			
1. Acetonitrile	53		ND
2. Acetonitrile + glucose	14		ND
3. Acetonitrile + glucose + ammonium	3		ND
4. Acetamide + glucose	0		29
5. Acetamide	0		78
6. Glucose + ammonium	0		20
(B) Induced			
1. Acetonitrile	73	39	1267
2. Acetonitrile + glucose	59	37	ND
3. Acetonitrile + glucose + ammonium	37	30	ND
4. Acetamide + glucose	14	14	ND
5. Acetamide	13	13	735
6. Glucose + ammonium	5	2	71

hydrolysed, unlike the corresponding nitriles, which did not act as substrates for the nitrile hydratase (Table 1).

All the nitriles and amides hydrolysed by intact cells of *R. erythropolis* BL1 were tested for their ability to serve as sole carbon and nitrogen sources for growth (Table 2). Simple aliphatic nitriles and amides supported growth, including unsaturated compounds such as allyl cyanide and 2-methyl 2-butenenitrile. Strain BL1 grew well on benzamide, whereas benzonitrile could not support growth.

Induction of NHA and AHA

The induction patterns of NHA and AHA were investigated by inoculating non-induced cells (A) and induced cells (B) into different growth media (Table 3). The

cells used as inocula were obtained from cultures pregrown for several passages in DE/glucose/ammonia medium and DE/acetonitrile medium, respectively. Non-induced cells regained 73 % of the maximum NHA after 24 h growth in DE/acetonitrile medium (Table 3, A1). When cells were grown in DE/acetonitrile medium containing additional carbon and/or nitrogen sources, much lower induction levels were observed (Table 3, A2 and A3). Acetamide did not induce NHA in strain BL1, either as a nitrogen source or when serving as sole carbon and nitrogen source (Table 3, A4 and A5). No NHA was detected in the control culture growing without nitrile (Table 3, A6).

Induced cells were tested for their ability to retain their NHA and AHA after transfer to growth media containing

different carbon/nitrogen sources (Table 3). Not surprisingly, the highest NHA [$73 \text{ U (g cell protein)}^{-1}$] was found in cells reinoculated into DE/acetonitrile medium (Table 3, B1). After the culture entered the stationary growth phase, the NHA decreased to 43% of the maximum level (Table 3, B1). The presence of additional carbon and nitrogen sources in the DE/acetonitrile medium significantly decreased expression of NHA in exponentially growing cells (24 h). The highest degree of repression was observed with cells grown in DE/acetonitrile medium supplemented with both glucose and ammonia (Table 3, B3). When induced cells were inoculated into a nitrile-free medium containing glucose and ammonia as sole carbon and nitrogen sources, the NHA decreased about 70% within 24 h (Table 3, B6). As seen from Table 3, acetamide did not induce NHA (Table 3, A5) although it somehow reduced repression of NHA in induced cells during subsequent growth in a nitrile-free medium (B4 and B5 vs B6). Cells lacking NHA always contained significant AHA even after many transfers in DE/glucose/ammonia medium (Table 3, A6). Even though the AHA of *R. erythropolis* BL1 could be increased manyfold when cells were grown in the presence of acetamide (or acetonitrile), the results clearly demonstrate the constitutive nature of the AHA as opposed to the inducible nature of NHA.

DISCUSSION

Five physiologically different nitrile-hydrolysing bacteria were isolated from marine environments on the east and west coast of Denmark. None of these isolates originated from areas close to nitrile-processing industries, suggesting that nitrile-hydrolysing bacteria are common inhabitants of marine sediments.

The ecological significance of nitrile-hydrolysing enzymes in both Gram-positive and Gram-negative bacteria is unknown. Are these enzymes a reminiscence of early prokaryotic life, when organic cyano compounds were part of the biosphere (Oró & Lazcano-Araujo, 1981; Ferris & Hagan, 1984; Loomis, 1988) – or are they produced in response to as yet unidentified organonitriles present in the environment? A number of naturally occurring nitriles have been identified (Jallageas, 1981). Since most of these are not commercially available they have not been tested as possible substrates for nitrile-hydrolysing enzymes. So far only a few biogenic nitriles (e.g. ricinine and mandelonitrile) have been reported to be hydrolysed by micro-organisms (Robinson & Hook, 1964; Yamamoto *et al.*, 1991). Inorganic cyanide (HCN/CN^-) is produced in vast amounts by natural synthesis (Vennesland *et al.*, 1981; Knowles, 1985) and is, therefore, of widespread occurrence in nature. However, known nitrile hydratases and nitrilases are unable to hydrolyse cyanide. Another explanation for the presence of nitrile-hydrating enzymes in bacteria could be that these enzymes have an unspecific enzymic reaction.

R. erythropolis strain BL1, described in this study, was isolated from a marine sediment which is exposed to salinities ranging from 2.4 to 3.4%. The strain grew in

the presence of 0–8% NaCl under laboratory conditions, with most rapid growth occurring with 1–3% NaCl (Fig. 1). *R. erythropolis* BL1 thus showed a relatively wide salt tolerance when grown on acetonitrile as sole carbon and nitrogen source. Other nitrile-hydrolysing bacteria belonging to the CMN-taxon (e.g. *Nocardia* spp., *Rhodococcus* spp., *Corynebacterium* spp.) do not appear to have been isolated from marine environments. However, subsequent studies may show that some of these isolates are also able to grow well in both freshwater and marine media.

R. erythropolis strain BL1 showed a much higher tolerance towards acetonitrile than reported previously for nitrile-hydrolysing bacteria. It grew well in media containing 900 mM acetonitrile (Fig. 3). Growth also occurred in DE medium containing 1.5 M acetonitrile ($\sim 8\%$, v/v), but with a long lag phase (unpublished results). In contrast, a *Rhodococcus* sp. studied by Miller & Gray (1982) grew slowly at 2% (v/v) but was virtually inhibited at 3%. A nitrile-hydrolysing strain of *Corynebacterium nitrilophilus* investigated by Mimura *et al.* (1969) tolerated up to 5% (v/v). It is possible that the high tolerance of *R. erythropolis* BL1 was a result of physiological adaptation occurring while the strain was routinely maintained on a medium containing 0.5% acetonitrile as sole carbon and nitrogen source.

The rate of acetonitrile hydrolysis (NHA) by resting cells of *R. erythropolis* BL1 also exhibited a high substrate tolerance, with maximum activity at 500 mM and with similar hydrolysis rates at 30 mM and 2 M. The NHA (and AHA) of strain BL1 were found to be 'cell-bound' – most likely in the cytoplasm (results not shown) which also seems to be the case for nitrile hydratase/amidase systems described in other bacteria (Miller & Knowles, 1984; Linton & Knowles, 1986). It would be interesting to know the concentration of acetonitrile present within the cells during nitrile hydrolysis at high external concentrations which are likely to be inhibitory to many intracellular enzymes. However, such data are not available to our knowledge.

Many studies on substrate specificity have been conducted with intact whole cells (e.g. DiGeronimo & Antoine, 1976; Arnaud *et al.*, 1980; Miller & Gray, 1982; Collins & Knowles, 1983; Ingvorsen *et al.*, 1988; Amarant *et al.*, 1989), which have clearly demonstrated that a large number of structurally different nitriles and amides can penetrate Gram-positive cells. Experiments performed with *Corynebacterium nitrilophilus* by Amarant's group (Amarant *et al.*, 1989) revealed no differences in the kinetic behaviour between whole cells, cell extracts and purified nitrile hydratase when testing small nitrile molecules. These data, therefore, suggest that the reaction is not limited by diffusion (Amarant *et al.*, 1989). Knowledge of the mechanism(s) of nitrile uptake by bacteria is, however, extremely limited (Miller & Knowles, 1984; Vaughan *et al.*, 1988; Amarant *et al.*, 1989). At present it cannot, therefore, be excluded that the nitrile hydratase/amidase enzyme system in the cytoplasm is exposed to substrate concentrations significantly lower than those of the

surrounding medium. If, however, Gram-positive cells are readily permeable to nitriles, high hydratase and amidase activities could be a means of maintaining low intracellular concentrations of toxic substrates and products.

Resting cells of *R. erythropolis* strain BL1 pregrown in acetonitrile medium were completely inactive towards benzonitrile and benzylocyanide (Table 1). Micro-organisms closely related to *R. erythropolis* BL1 (e.g. *N. rhodochrous* NCIB 11216, *Brevibacterium* R312, *N. rhodochrous* LL100-21, *R. erythropolis* CH5 and *C. nitrilophilus*) have been reported to hydrolyse a number of aromatic nitriles such as benzonitrile, benzyl cyanide and cyanopyridines either by means of a broad-spectrum nitrile hydratase or a nitrilase specific for aromatic nitriles (Harper, 1977; Arnaud *et al.*, 1977; Collins & Knowles, 1983; Ingvorsen *et al.*, 1988; Amarant *et al.*, 1989). A nitrile hydratase also lacking activity towards aromatic nitriles was reported in *N. rhodochrous* LL100-21. This strain was, however, able to induce a separate nitrilase enzyme for conversion of benzonitrile directly to benzoate after cultivation in a benzonitrile-containing medium (Collins & Knowles, 1983). Growth of *R. erythropolis* BL1 in DE/acetonitrile medium supplemented with benzonitrile or benzylocyanide did not result in the induction of enzyme activities towards these compounds.

The data in Table 3 show that expression of nitrile hydratase and amidase activities in strain BL1 were not closely coupled, which probably means that the structural genes coding for NHA and AHA are located on different operons. In *R. erythropolis* BL1, NHA was not induced by acetamide, although it appeared to reduce repression of NHA (Table 3, B4 and 5). These results are clearly at variance with those obtained with *Rhodococcus* sp. and *Nocardia rhodochrous* LL100-21, where acetamide was found to be a powerful inducer of the nitrile hydratases (Miller & Gray, 1982; Collins & Knowles, 1983). It was suggested that the structural genes for NHA and AHA in these two micro-organisms were located on the same operon and expressed concurrently, e.g. after induction by acetamide (Linton & Knowles, 1986; Mayaux *et al.*, 1990; Ikehata *et al.*, 1989). Later studies did indeed show nitrile hydratases and amidases to be present on the same operon (Kobayashi *et al.*, 1992, 1993).

The 'cell-bound' nitrile hydratase/amidase system of *R. erythropolis* BL1 was extremely tolerant to high concentrations of acetonitrile (Figs 3 and 4). Due to this property and its broad substrate specificity the nitrile hydratase/amidase enzyme system of strain BL1 may be of potential use for synthesis or detoxification processes.

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Received 6 April 1995; revised 1 September 1995; accepted 19 September 1995.