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A *Pseudomonas putida* capable of stereoselective hydrolysis of nitriles

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Abstract *Pseudomonas putida* NRRL-18668 contains a nitrile hydratase capable of stereoselective hydrolysis of 2-(4-chlorophenyl)-3-methylbutyronitrile at more than 90% enantiomeric excess (ee) to the (*S*)-amide. This soil isolate was recovered from enrichments using (*R,S*)-2-methylglutaronitrile as the sole nitrogen source. Enzyme expression is constitutive and does not show a high level of catabolite repression. The organism is capable of growth on a wide variety of aliphatic mono- and dinitrile compounds. The hydrolysis activity on propionitrile is approximately 10.3 μmole h^{-1} mg wet $cells^{-1}$. The enzyme in cell-free preparations is inhibited by a number of heavy metals, phenylhydrazine, and cyanide. Substrate specificity is broad with highest rates shown on C_4 and C_5 aliphatic mononitriles. The strain appears somewhat unusual in its dependence on cobalt supplementation for maximum enzyme activity and the ability to hydrolyze some aromatic nitriles. This strain is also capable of a two-step hydrolysis of 2-(4-isobutylphenyl)-propionitrile and 2-(6-methoxy-2-napthyl)-propionitrile to the (*S*)-acids (ibuprofen and naproxen respectively) with stereoselectivity residing primarily in the aliphatic amidase. This appears to be the first description of a steroselective nitrile hydratase from a gram-negative organism.

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Introduction

Nitrile hydratases, catalyzing the reaction R-CN + H_2O → $RCONH_2$, make up one broad enzyme group capable of nitrile (R-CN) hydration. A wide range of gram-negative and gram-positive strains containing these enzymes has been described (Nagasawa and Yamada 1990; Novo et al. 1995). In addition, all nitrile-hydratase-containing organisms so far described also contain aliphatic amidases, which catalyze the reaction $RCONH_2$ → RCOOH + NH_3 and are expressed in coordination with the hydratase (Arnaud et al. 1976; Ciskanik et al. 1995; Kobayashi et al. 1993). The catalytic mechanisms are not completely understood for any of these enzymes. However, nitrile hydratases are generally characterized by the presence of a coordinated metal, iron or cobalt (Nagasawa and Yamada 1989, 1990) or, recently, both (Kobayashi et al. 1995). In contrast, amidases are thought to be dependent on thiol groups for their catalytic activity and, with one exception (Nawaz et al. 1994), are not reported to contain coordinated metals (Novo et al. 1995; Tata et al. 1994).

Nitrile hydrolysis for biocatalytic chemical synthesis is currently a very active research area (Kobayashi et al. 1992). Early reports on nitrile hydratases and their applications come from groups in both France (Arnaud et al. 1976; Bui et al. 1984) and Japan (Nagasawa et al. 1987, 1991). Production of acrylamide by the Nitto Chemical Co. is the best current example of a fully developed industrial application (Kobayashi et al. 1992). Numerous suggestions have also been made for other applications in the production of pharmaceuticals and fine chemicals (e.g., Eyal and Charles 1990; Vo-Quang et al. 1987). We were the first to report the occurrence of a nitrile hydratase enzyme from *Pseudomonas putida* NRRL-18668 with high stereoselective activity on 2-(4-chlorophenyl)-3-methylbutyronitrile (Anton et al. 1992). Recent reports demonstrate stereoselective nitrile hydrolysis in two *Rhodococcus* strains (Blakely et al. 1995; Layh et al 1994). However, *P. putida* NRRL-18668 ap-

pears to be the only gram-negative organism so far reported with a steroselective nitrile-hydratase. Further characterization of this nitrile-hydratase-containing organism has shown other interesting features.

Materials and methods

Enrichment, isolation, fermentation

Mineral salts medium used for enrichments, maintenance and fermentations included, per liter: 63 ml 1 M sodium phosphate buffer plus 0.05 g $FeSO_4.7H_2O$, 0.5 g $MgSO_4.7H_2O$, and 0.24 g sodium citrate, pH 7.2. This was supplemented with 1.0 ml trace element solution SL-7 (ATCC 1989). Yeast extract, 10 mg/l, was included for maintenance and fermentations while 1 ml/l Wolfe's vitamin solution (ATCC 1989) was used for enrichment media. Enrichment was done at room temperature with glucose (10 g/l) as the carbon and energy source and (*R*,*S*)-2-methylglutaronitrile (MGN), 25 mM as the nitrogen source. Samples for enrichment were collected from soil at a nylon intermediates plant in Orange, Tex. Following four passes through MGN medium, the cell suspension was plated on MGN medium with 2% Noble agar. Selected colonies were picked and tested for growth and ammonia evolution in MGN liquid medium. Clones chosen for further testing were grown in MGN medium and stored frozen at −70 °C in a 15% glycerol suspension. Fermentations were run in a Braun Biostat E 10-l fermenter at 25 °C, 80% O_2 saturation with agitation at 400–1000 rpm using the basal medium described above with 10 mM propionitrile as the nitrogen source. Following growth, cells were harvested by centrifugation in the cold and washed once in cold 0.1 M phosphate-buffered saline, pH 7.2, with 15% glycerol. Washed cells were quick frozen on solid CO_2 and then stored at −65 °C for later use.

Strain/enzyme characterization

The isolate was taxonomically characterized by various methods, involving (1) standard biochemical test media: Oxi-Ferm, Enterotube II (both, Roche Diagnostics, N.J.) and (2) the American Type Culture Collection (Rockville, Md.). Growth on nitriles or amides as nitrogen or carbon/energy + nitrogen source was tested by combining substrates at 10 mM concentration in basal medium with or without glucose. Growth compared to control samples (both no nutrient and no inoculum) was followed turbidimetrically.

Propionitrile (10 mM) hydrolysis in a 3-ml reaction of 0.1 M phosphate-buffered saline, pH 7.2, with 2 µl cell-free enzyme (see below) preparation was used to evaluate inhibitor susceptibility, the K_m for propionitrile and substrate preferences. Temperature optimum and aryl-2-alkane nitrile and amide hydrolysis experiments used whole, resting cells in a similar format. The incubation buffer was 0.1 M, pH 7.2 phosphate buffer, except in the enzyme-inhibition experiments where pH was adjusted by acid/base additions to pH 6.8–7.1. Unless otherwise indicated, assays were run at 25 °C. Cells were suspended at 1–10 mg wet weight/ml. Unless otherwise indicated, samples generally were collected at 2, 5, 10, 20, and 60 min in standard assays with aliphatic nitriles.

Cell-free enzyme was prepared by breaking cells in EDBTC buffer: 2 mM EDTA, 5 mM dithiothreitol, 40 mM butyrate in 20 mM TRIS/HCl, pH 7.3. A 50-g sample of frozen cells was suspended in buffer at 0.17 g/ml and sonicated at 4 °C. Cell debris was removed by centrifugation at 24 000 *g*, 30 min, 4 °C. The protein fraction precipitating between 45% and 60% NH_4SO_4 was collected by centrifugation at 24 000 *g*, 30 min, 4 °C. Protein was dialyzed against EDBTC buffer and stored at 4 °C for later use. This stock was diluted 30× to 50 µg protien/ml in appropriate buffer for testing.

In both resting cell and cell-free preparations, enzyme activity was routinely monitored by measuring the amount of methacrylonitrile-hydrolyzing activity present via a spectrophotometric assay. The rate of increase in absorption at 224 nm was used as an indicator of conversion of methacrylonitrile to methacrylamide. An aliquot of the material to be tested was suspended in 0.1 M HEPES [*N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid)] buffer, pH 7.1, at twice the test concentration (generally, 1–10 mg/ml for whole cells). Sample and substrate were brought to the assay temperature of 25 °C. Methacrylonitrile, 20 mM in 0.1 M phosphate buffer, pH 7.1, was mixed with an equal volume of 2× sample (= 10 mM methacrylonitrile final concentration). At each assay time, activity was stopped by the addition of 10% phosphoric acid, which brought the pH to 2.5–3 in the reaction mixture. Solids were removed by centrifugation and the absorption at 224 nm was measured. Units of activity were calculated according to the equation:

$$\text{Activity(IU)} = \frac{(\Delta A_{224/\text{min}}) \times (\text{assay volume, l}) \times 10^6 (\mu\text{mol/mol})}{(3400\ \text{l mol}^{-1}\text{cm}^{-1}) \times 1\ \text{cm pathlength}}$$

Analytical

C_2–C_6 nitriles and their amide and acid hydrolysis products were monitored by gas chromatography on an HP 5880A with flame-ionization detector. A Nukol (Supelco, Bellefonte, Pa.), 0.53-mm wide-bore capillary column with helium flow at 2.5 ml/min, using a temperature program of 80–220 °C at 25 °C/min then holding at 220 °C for 2–10 minutes, gave an elution order of nitrile, acid, amide from a 3.3-µl aqueous injection (acidified to pH 2.5–3.0 with 10% phosphoric acid). The final hold time was adjusted for differing product volatilities.

Aryl-2-alkane nitriles and their hydrolysis products were monitored on a Zorbax 4.6-mm × 150-mm C_{18} reverse-phase HPLC column with UV detection (220–240 nm). The eluant was methanol/0.1% H_3PO_4 (7:3) or acetonitrile/0.1% H_3PO_4 (67:33) at 1–2.5 ml/min flow. Chiral products were resolved on an α_1-acid glycoprotein column (Chromtech, Sweden) with UV detection. Eluents were as follows: 2-(4-chlorophenyl)-3-methylbutyronitrile (CPIN), amide (CPIAm), and acid in 0.01 M phosphate buffer (pH 6.0)/ethanol (19:5 v/v); 2-(6-methoxy-2-napthyl)-propionitrile (NPCN), amide (NPAm), and acid (NPAC) in 0.01 M phosphate buffer (pH 5.6)/ethanol (19:5 v/v); 2-(4-isobutylphenyl)-propionitrile (IBCN), amide (IBAm) and acid (IBAC) in 0.02 M phosphate buffer (pH 5.2)/ethanol (24:1 v/v); attrolactonitrile (2-phenyl-2-hydroxy-propionitrile (ATCN), amide and acid in 0.01 M phosphate buffer (pH 6.0)/ethanol (19:5 v/v). Enantiomer ratios are expressed as *S*/*R* or as enantiomeric excess (% ee) = (major enantiomer − minor enantiomer)/(total enantiomers).

Chemicals

All nitriles and amides used in growth/activity experiments were obtained from Aldrich Chemcial Co. or Fluka Chemcial Co. at +97% purity or the highest available lesser purity. IBCN and NPCN were synthesized from *p*-isobutylstyrene and 2-methoxy-6-vinylnaphthalene, respectively (Nugent and McKinney 1985). ATCN was synthesized as the acetophenone cyanohydrin from the stable *O*-trimethylsilyl derivative. CPIN was obtained from Sumitomo Chemical Co. The (*S*)-NPAm and (*S*)-IBAm were synthesized from the corresponding (*S*)-acid via the acid chloride.

Results

A variety of bacterial and fungal isolates was obtained from the enrichments of environmental material. On the basis of high specific activity for conversion of (*R*,*S*)-CPIN to (*S*)–CPIAm, the isolate 5B-MGN-2p was chosen for further study. The isolate is gram-negative and

oxidase-positive. Biochemical testing by ATCC yielded a pattern characteristic of *P.* putida of indeterminate affiliation with biovars A or B (Stanier et al. 1966). The strain is deposited at the US Department of Agriculture Northern Regional Research Center as NRRL-18668.

Nitrile hydratase expression levels were relatively stable throughout a batch-fermentation growth cycle on nitrogen-limited (available C/N ≈ 33/1) basal medium with glucose plus ammonia, showing a specific activity of 15 nmol (*S*)-CPIN h^{-1} / mg wet $cells^{-1}$ 1 h into the run and increased to only 18 nmol (*S*)-CPIN h^{-1} / mg wet $cells^{-1}$after 17 h.

Stereoselectivity remained constant. The 10-l fermentation yielded 18.2 g wet weight/l with a maximum observed doubling time of 1.6 h. Additional observations suggest that enzyme expression is constitutive. The isolate shows nitrile hydratase activity of similar magnitude in the presence or absence of nitriles. Tests of different medium formulations showed that enzyme-specific activity in whole-cell preparations was not strongly affected by growth conditions in 1-l batch flasks. Cells grown to late log with glucose as the carbon/energy source under nitrogen-limited conditions with 5 mM nitrogen source showed the following relative specific activity levels: urea 100 % (77 μmol methacrylonitrile min^{-1} mg dry weight $cells^{-1}$), methacrylonitrile 94 %, crotononitrile 92 %, NH_4Cl 65 %, valeronitrile 52 %, $NaNO_3$ 45 %. A wider range of effects due to the nitrogen source was observed on the doubling times: NH_4Cl 2.6 h, valeronitrile 2.6 h, urea 2.7 h, $NaNO_3$ 3 h, methacrylonitrile 3.1 h, crotononitrile 6.5 h. Under nitrogen-limited conditions with urea as the nitrogen source, the carbon/energy source only had a moderate influence on the relative nitrile-hydratase-specific activity: succinate 100 % (131 μmol methacrylonitrile min^{-1} mg dry weight $cells^{-1}$), pyruvate 99 %, glycerol 87 %, glucose 77 %. Growth under similar conditions with ammonium chloride as the nitrogen source resulted in the following relative activities: glycerol 100 % (101 μmole methacrylonitrile min^{-1} mg dry weight $cells^{-1}$), pyruvate 100 %, succinate 90 %, glucose 77 %. Doubling times were also little influenced by carbon source, ranging from a maximum of 2.2 h for glucose and succinate to a minimum of 3.5 h for glycerol across both urea and ammonium chloride treatments.

The 5B isolate showed metabolic flexibility in the ability to use nitriles as sources of nitrogen and carbon/energy for growth. Many mono- and dinitriles, substituted aliphatic nitriles and aromatic nitriles could serve as nitrogen sources for growth: chlorobutyronitrile, fluoroacetonitrile, 2-pentenenitrile, trimethylacetonitrile, 2–phenylbutyronitrile, acetonitrile. A variety of aliphatic and aromatic nitriles served as both nitrogen and carbon/energy sources for growth: adiponitrile, benzonitrile, butyronitrile, chlorovaleronitrile, crotononitrile, 3-cyanopyridine, dicyanohexane, hexanenitrile, isovaleronitrile, methacrylonitrile, 2-methylbutyronitrile, 2-methylglutaronitrile, pelargononitrile, propionitrile, valeronitrile. The ability to grow on a variety of nitriles implies the presence of complimentary amidase activity.The use of five different amides as a source of nitrogen in the presence of glucose was explicitly demonstrated: isobutyramide, acetamide, propionamide, benzamide, nicotinamide. Growth did not occur on acrylonitrile, but in whole-cell assays (5 mg wet cells/ml, 10 mM acrylonitrile) acrylonitrile was hydrolyzed with first-order kinetics ($\tau_{1/2}$= 4.7 min) and acrylamide accumulated. A number of other R-CN compounds, when used as a nitrogen source would not support growth: chloroacetonitrile, cyanide, 4-cyanophenol, 3-indolylacetonitrile, isobutyronitrile, lactonitrile, napthylacetonitrile, phenylacetonitrile, α-phenylcinnamonitrile.

Whole, resting-cell preparations of the 5B isolate hydrolyzed some aryl-2-substituted, aliphatic nitriles and amides (Table 1). In all cases where conversion occurred, it was stereoselective. In the case of CPIN, the nitrile hydratase showed a strong preference for (*S*)-enantiomer hydrolysis. The hydrolysis time course indicated that this was a kinetic preference and was not absolute (Fig. 1) Upon conversion of at least 90 % of the *S* enantiomer, *R* enantiomer conversion continued but at a 6.2-fold slower rate (Fig. 1). Amide *S*/*R* enantiomeric ratios of at least 20 were observed (Fig. 1, Table 1) for substrate conversion above 90 %. Hydro-

Table 1 Enantiomer preferences for nitrile hydratase and amidase activities on aryl-2-alkane substrates for *Pseudomonas putida. CPIN, CPIAm*, 2-(4-chlorophenyl)-3-methylbutyronitrile and amide; *NPCN, NPAm*, 2-(6-methoxy-2-napthyl)-propionitrile and amide; *IBCN, IBAm*, 2-(4-isobutylphenyl)-propionitrile and amide; *ATCN, ATAm*, 2-phenyl-2-hydroxy-propionitrile and amide. *NC* no hydrolysis detected in 48 h

Substrate	ATCN or ATAm	CPIN or CPIAm	NPCN or NPAm	NPAm	IBCN or IBAm
Nitrilehydratase					
Temperature (°C)	28	28	28	–	28
Conversion(%)	NC	56	29	–	29
Preferred enantiomer	NC	*S*	*R*	–	*R*
Product ratio, *S*/*R*	NC	>20	0.25[a]	–	<0.05[a]
Amidase					
Temperature(°C)	28	28	28	50	28
Conversion(%)	NC	NC	60	18	12
Preferred enantiomer	NC	NC	*S*	*S*	*S*
Product ratio, *S*/*R*	NC	NC	4	>20	>20

[a] Indicates that *S*/*R* ratio is influenced both by nitrile hydratase and amidase selectivity

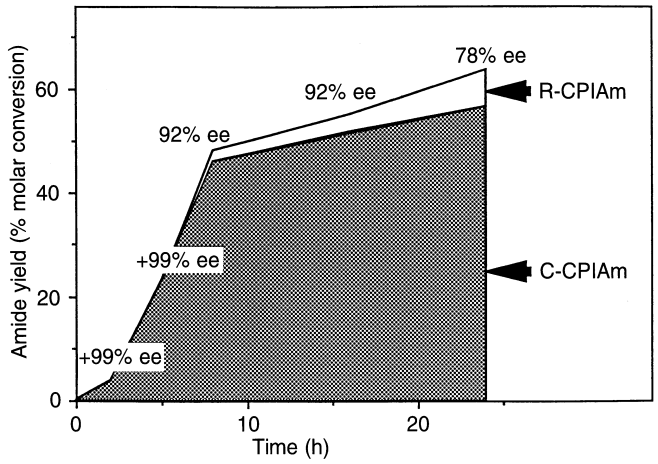

Fig. 1 Hydrolysis of (*R*,S)-2-(4-chlorophenyl)-3-methylbutyronitrile at 25 °C by whole resting cells of the 5B isolate. Values at top indicate the percentage enantiomeric excess [*ee* = (*S*−*R*)/(*S*+*R*)] of 2-(4-chlorophenyl)-3-methylbutyronitrile amide (*CPIAm*) at 3, 5, 8,16, and 24 h

lytic activity and enantiomeric discrimination varied for different aryl-2 substituted nitriles. Hydrolysis of NPCN and IBCN showed an *R* enantiomer preference with an *S*/*R* enantiomeric ratio of 0.25 (= 4, *R*/*S*) and below 0.05 (>20, *R*/*S*) respectively, at conversion values of 29% (Table 1). The cyanohydrin, attrolactonitrile, was not hydrolyzed by the 5B enzyme(Table 1). Resting-cell preparations also showed an *S* enantiomer preference during hydrolysis of NPAm and IBAm (Table 1). NPAm hydrolysis occurred at both 28 °C and 50 °C. Higher rates of conversion resulted in lower enantiomer ratios in the products, suggesting that kinetic resolution was occurring (Table 1).

Cell-free nitrile hydratase showed a strong substrate preference for linear aliphatic nitriles of the C_4–C_5 length. For a selected group of nitriles, relative first-order hydrolysis rates (%, where propionitrile = 100%) were acetonitrile <1, isobutyronitrile 2, hexanenitrile 3, isovaleronitrile 5, trimethylacetonitrile 23, benzyl cyanide 28, methacrylonitrile 29, adiponitrile 29, fluoroacetonitrile 40, benzonitrile 97, *cis*-2-pentenenitrile 115, 4-chlorobutyronitrile 226, butyronitrile 383, chloroacetonitrile 386, and valeronitrile 522. The K_m for propionitrile in the cell-free preparation was 48 mM. In addition, specific turnover rates for resting, whole cells at 10 mM substrate concentration were substantially higher for propionitrile: 10.3 μmol h^{-1} mg wet $cells^{-1}$, compared to the racemic, aryl-2-substituted, aliphatic nitrile, (*R*,*S*)-CPIN, 0.011 μmol h^{-1} mg wet $cells^{-1}$.

Propionitrile hydrolysis in whole resting cells showed a typical temperature response with an optimum of about 30 °C. Rates dropped to 1/3 maximum at 10 °C and enzyme activity was undetectable after 10 min at 50 °C. A number of chemical inhibitors were effective at inactivating the cell-free enzyme. In the presence of 1 mM concentrations of Cd, Cu, Hg, Ag, and Zn, as well as phenylhydrazine, methacrylonitrile hydrolysis rates were reduced to less than 20% of the control. Sodium cyanide at 1 mM caused a reduction to less than 30% of the control. In contrast, iodoacetic acid, iodoacetamide, NaN_3, EDTA, cysteamine, urea, 1,10-phenanthroline, and H_2O_2, all at 1 mM concentration, caused little or no inhibition with rates exceeding 85% of the control.

Cobalt supplementation to 100 μM in the minimal medium caused a 3-fold increase in the length of the lag phase. However, it significantly increased specific nitrile hydratase activity. On a constant-wet-weight basis, the hydrolysis rate for methacrylonitrile increased 44-fold. Rates of CPIN hydrolysis also increased with cobalt supplementation while typical stereoselectivity was retained. Hydrolysis rates (based on amide appearance) were: supplemented, 0.38 μmol (*S*)-CPIN/h, 0.043 μmol (*R*)-CPIN/h; unsupplemented, 0.24 μmol (*S*)-CPIN/h, <0.01 μmol (*R*)-CPIN/h. Mixed enantiomer hydrolysis increased from 0.04 μmol/h to 0.32 μmol amide/h with supplementation.

Discussion

Nitrile hydratases have been described from a number of gram-positive and gram-negative species. However, there have been only two other reports of these enzymes with stereoselectivity greater than 80% ee. Although numerous descriptions exist in the patent literature of stereoselective, whole-cell biocatalysts for acid production from nitriles (e.g., Jallegeas et al. 1982; Yamamoto et al. 1994), careful analysis usually reveals that the stereoselectivity resides primarily in the amidase, not in the hydratase (Gilligan et al. 1993; Matsutomo et al. 1995). Blakely et al. (1995) have reported production of (*R*)-(+)-2-phenylbutyramide at an enantiomeric excess of 83%. The 2-phenylbutyramide was not a substrate for the amidase. Therefore, accumulation of the amide intermediate allowed easier recognition of the stereoselective nitrile hydratase activity (Blakely et al. 1995). In the presence of diethylphosphorimidate for amidase inactivation, *Rhodococcus* sp. C3II produces (*S*)-NPAm of 94% ee at 30% conversion from (*R*,*S*)-NPCN (Layh et al. 1994). The nitrile hydratase from *P. putida* NRRL-18868 yields more than 95% ee of (*S*)-CPIAm at conversions below 25%, dropping to about 92% ee near 50% (*R*,*S*)-CPIN conversion. From Fig. 1 and experiments using enantiomeric CPIN, it is clear that a kinetic resolution is occurring with the enzyme showing an almost ten-times kinetic preference for the (*S*)-CPIN enantiomer. Since the *S*-enantiomer prevents entry of the *R*-enantiomer into the active site, as the reaction proceeds and the concentration of (*S*)-CPIN declines, the relative rate of *R*-CPIN hydrolysis rises. (*S*)-IBAC (ibuprofen); above 90% ee, and (*S*)-NPAC (naproxen), above 90% ee, are also produced by *P. putida* NRRL-18868 from the racemic nitriles, but in these cases both the nitrile hydratase and amidase activities contribute to the enantiomeric excess in the acid product. There was

also a strong temperature effect observed on amidase stereoselectivity with (*R*,*S*)-NPAm, with the (*S*)-NPAC having a 60 % ee at 28 °C and above 90 % ee at 50 °C. On the basis of the initial rate of appearance, the *P. putida* nitrile hydratase shows slight preference for the *R*-enantiomers of both IBCN and NPCN. Attrolactonitrile and attrolactamide were not hydrolyzed. Considering also the failure to use lactonitrile as a nitrogen source, it appears that the hydratase is not well-suited for hydrolysis of α-hydroxy nitriles.

Nitrile hydratases have been reported from a number of different *Pseudomonas* species (e.g. Matsutomo et al. 1995; Chapatwala et al. 1993; Yanase et al. 1985). The best-described nitrile-hydaratase-containing *Pseudomonas* strain is *Pseudomonas chlororaphis* B23 (FERM-187) developed by Yamada and co-workers for acrylamide production (Kobayashi et al. 1992). *P. putida* 5B-MGN-2p (NRRL-18668) shows a number of differences compared to this strain. *P. putida* shows no evidence for enzyme induction. A range of nitrile inducers and urea failed to elicit a significant increase in nitrile-hydratase-specific activity. In contrast, the B23 strain shows induction in the presence of methacrylamide or isobutyronitrile and, in an optimized medium with iron and amino acid supplementation containing 0.5 g/l methacrylamide, nitrile hydratase activity was enhanced 900 times over the activity in basal medium (Nagasawa et al. 1989). The minor differences in relative specific activity seen for different carbon/energy sources also suggests that enzyme synthesis is not strongly influenced by catabolite repression. The 5B strain requires 100 μM cobalt for maximal nitrile hydratase activity. This may be an induction phenomenon or indicate that cobalt serves in the enzyme active sight. The B23 strain with an iron enzyme (Nagasawa et al. 1987), has not been reported to require cobalt supplementation, although such a requirement has been reported for *Rhodococcus rhodochrous* J1, a strain with a cobalt enzyme (Nagasawa et al. 1991). Growth on a wide variety of nitriles as carbon and nitrogen sources also demonstrates wide-spectrum nitrile hydratase and amidase activity in combination with the ability to feed a variety of carbon sources into central metabolism. We have also found that this enzyme will hydrolyze nitrile groups on a number of relatively large (Formula Weight ≤ 389), complex, proprietary compounds. These observations are consistent with a relatively broad substrate specificity for the enzyme.

Although some nitrile-hydrolyzing enzymes are plasmid-derived in gram-negative organisms (Stalker and McBride 1987), previous examples from pseudomonads, when examined, have been chromosomally derived (Yanase et al. 1985; Nishiyama et al. 1991). This appears to be the case for *P. putida* NRRL-18868 where no evidence for covalently closed circular DNA could be found following a standard alkaline-lysis procedure (Sambrook et al. 1989).

Although unusual in its stereoselectivity, the *P. putida* nitrile hydratase shows many typical features. A wide substrate spectrum for aliphatic nitriles and dinitriles is common to many nitrile hydratases (Bui et al 1984; Blakely et al. 1995). K_m values for short-chain mononitriles are typically in the 10–100 mM range (Bui et al. 1984). Since nitrile hydratases typically use straight-chain compounds (Nagasawa and Yamada 1989), the ability to hydrolyze benzonitrile is somewhat unusual for an aliphatic nitrile hydratase, but not without precedence (Nagasawa et al. 1991). The presence of an aromatic amidase to complement this activity is also unusual, but has been reported for a *Pseudomonas* sp. (Kagayama and Ohe 1990). Whole-resting-cell assays also showed that regiospecific hydrolysis occurs with some dinitriles: adiponitrile preferentially converted to 5-cyanovaleramide and 1,3-dicyanobenzene also was converted to the cyanoamide (data not shown). The behavior of the cell-free nitrile hydratase shows features common to many such hydratases (Bui et al. 1984; Nagasawa et al. 1989, 1987). The optimal temperature is about 30 °C with rapid inactivation occurring above 35 °C. A number of heavy metals as well as cyanide cause strong inhibition, whereas organic sulfhydryl inhibitors or metal chelators cause much less inhibition. The typical nitrile hydratase active-site metal is thought to be part of a cysteine complex (Jin et al. 1993), perhaps sensitive to heavy metal replacement.

P. putida 5B-MGN-2p (NRRL-18668) appears to contain an unusual nitrile hydratase capable of stereoselective hydrolysis, which may also require cobalt at its active site. To our knowledge, this is the only gram-negative strain known to contain a nitrile hydratase capable of highly stereoselective hydrolysis. This enzyme has the potential for wide industrial application with its broad substrate specificity and demonstrated stereoselective and regioselective capabilities. Work is continuing to better characterize the enzyme as well as to improve its capabilities for industrial processes.

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