Novel thermophilic bacteria producing nitrile-degrading enzymes

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The first known report of the isolation of thermophilic bacteria which produce nitrile-degrading enzymes is presented. One of the strains isolated was studied in detail. Strain Dac521, classified as *Bacillus pallidus*, was capable of growth on acetonitrile, benzonitrile, propionitrile, acetamide, benzamide and propionamide as the sole carbon and nitrogen source in minimal nutrient media. The strain produced separate aliphatic-nitrile (e.g. acetonitrile)- and aromatic-nitrile (e.g. benzonitrile)-degrading activities. Acetonitrile-degrading activity was produced constitutively and enzyme production was not enhanced by the addition of substrate. Under conditions where benzonitrile was the sole carbon and nitrogen source in minimal nutrient media, acetonitrile-degrading enzyme activity was completely inhibited and benzonitrile-degrading activity was induced. Growth on substrates as sole carbon and nitrogen sources, together with the substrate specificity of cell-free extracts, suggested that acetonitrile and benzonitrile degradation may have occurred via nitrile hydratase and nitrilase pathways, respectively. Both the acetonitrile- and benzonitrile-degrading enzyme systems were significantly more thermostable in whole-cell preparations and cell-free extracts compared to their mesophilic counterparts.

Keywords: *Bacillus pallidus*, thermophilic, nitrilase, nitrile hydratase

INTRODUCTION

Nitriles (i.e. organo-cyanides of the general formula $RC\equiv N$) are common constituents of plants (Kobayashi *et al.*, 1989) and occur as intermediates of microbial metabolism (Kobayashi *et al.*, 1993a). Their hydrolysis is catalysed by nitrile-degrading enzymes which occur in a wide variety of plants (Thimann & Mahadevan, 1964), mesophilic bacteria [e.g. *Brevibacterium* (Arnaud *et al.*, 1977), *Arthrobacter* (Asano *et al.*, 1982), *Nocardia* (Harper, 1985), *Klebsiella* (Stalker *et al.*, 1988), *Corynebacterium* (Li *et al.*, 1992) and *Rhodococcus* (Langdahl *et al.*, 1996)] and fungi [e.g. *Fusarium solani* (Harper, 1977), *Myrothecium verrucaria* (Maier-Greiner *et al.*, 1991) and *Fusarium lateritium* (Cluness *et al.*, 1993)].

Nitrile hydrolysis occurs by two major enzymic pathways (Faber, 1992; Mahadevan & Thimann, 1964). One pathway (1) involves the sequential hydrolysis of the nitrile molecule to its corresponding carboxylic acid and ammonia via an amide intermediate, catalysed by two different enzymes: a hydratase and an amidase. The other (2) is the direct hydrolysis by a nitrilase to the corresponding carboxylic acid and ammonia.

These enzymes are of particular interest as many nitriles and their hydrolysed derivatives are used in commercial processes including paper manufacture (Hwang & Chang, 1989) and waste treatment (Nazly *et al.*, 1983) or to produce commercially viable compounds such as acrylamide (Kobayashi *et al.*, 1993b), antibiotics (Jallageas *et al.*, 1980), anti-inflammatory agents (Gilligan *et al.*, 1993) and herbicides (Bianchi *et al.*, 1991). Chemical hydrolysis of nitriles often involves relatively harsh conditions which precludes the use of nitriles carrying sensitive functionalities. In addition, unwanted by-products, including large amounts of salt, are often formed. Biotransformation of nitriles, using nitrile-degrading enzymes (Bengis-Garber & Gutman, 1988; Bhalla *et al.*, 1992), overcomes these problems and may offer the additional advantage of stereospecificity (Bianchi *et al.*, 1991; Kakeya *et al.*, 1991; Layh *et al.*, 1992). However, application of these enzymes in in-

dustry is still limited, in part due to the intrinsic thermal instability (Nagasawa *et al.*, 1993) of the known mesophilic enzymes. Here we report the isolation of thermophilic nitrile-degrading bacterial strains, together with evidence that the intracellular nitrile-degrading enzymes have substantially higher thermal stability than known mesophilic homologues.

METHODS

Chemicals. Acetonitrile and acrylamide were obtained from BDH. Acrylonitrile, benzamide, benzonitrile, isovaleronitrile, propionamide and propionitrile were obtained from Aldrich. Dithiothreitol and acetamide were obtained from Sigma-Aldrich. Nutrient broth no. 2 and purified agar were obtained from Oxoid. API 20E and API 50CH analytical profile index kits and API 50 CHB media were obtained from BioMérieux. Working concentrations of benzonitrile were prepared in 10% (v/v) methanol.

Microbial culture. Cultures were grown on minimal nutrient plates at 50 °C for up to 7 d. The minimal nutrient plates contained: (NH₄)₂SO₄, 1.60 g l⁻¹; MgSO₄, 0.25 g l⁻¹; CaCl₂, 0.05 g l⁻¹; KH₂PO₄, 0.50 g l⁻¹ (pH 7.5); purified agar, 20.00 g l⁻¹; and 0.10% (v/v) nitrile. Five different nitriles were used separately as the sole carbon source: acetonitrile, benzonitrile, isovaleronitrile, propionitrile and acrylonitrile.

Pure isolates were grown in liquid minimal media using each of the five nitriles separately as the sole nitrogen source. The minimal media contained: MgSO₄, 0.25 g l⁻¹; CaCl₂, 0.05 g l⁻¹; KH₂PO₄, 0.50 g l⁻¹; CoCl₂, 0.35 g l⁻¹; 0.50% (v/v) glycerol; and 0.05% (v/v) nitrile.

Isolate Dac521 was routinely grown in the following minimal medium: KH₂PO₄, 2 g l⁻¹; NaCl, 1 g l⁻¹; MgSO₄, 0.2 g l⁻¹; thiamin, 0.4 mg l⁻¹; biotin, 2 µg l⁻¹; inositol, 2 mg l⁻¹; FeSO₄, 10 mg l⁻¹; (NH₄)₂SO₄, 1.32 g l⁻¹; sodium succinate, 5.4 g l⁻¹; pH 7.2. Nitriles (20 mM) were substituted for succinate and/or (NH₄)₂SO₄ as the sole carbon and/or nitrogen source, respectively, except where otherwise stated. Cultures were grown at 50 °C in 250 ml flasks containing 50 ml media or in 2 l flasks containing 500 ml media, with a reciprocation rate of 300 r.p.m.

Strain characterization. Standard oxidase tests, spore staining, Gram staining, catalase tests and motility tests (Collins *et al.*, 1995) were performed using fresh cultures of strain Dac521.

Analytical profile index (API) tests 50CH and 20E (Logan & Berkeley, 1984) were used to aid identification. API 50 CHB defined medium and distilled water were used to inoculate the test kits, respectively. Both 50CH and 20E API strips were incubated at 50 °C and the experiments were performed in triplicate according to the protocol set out in the API guidelines.

For hybridization studies, chromosomal DNA was extracted from test strains using the method of White *et al.* (1993). DNA from strain Dac521 was labelled with [³²P]dCTP by the random primer method and unincorporated nucleotides were removed using NAP-10 columns. Probe DNA was hybridized to target DNA from 10 thermophilic *Bacillus* species (*B. thermoglucosidasius*, *B. thermodenitrificans*, *B. caldovelox*, *B. stearothermophilus*, *B. pallidus*, *B. caldolyticus*, *B. kaustophilus*, *B. smithii*, *B. thermocloacae* and *B. thermo-*

catenulatus; kindly provided by Professor R. Sharp, CAMR, Porton Down, UK) immobilized on nitrocellulose filters using a slot blot system (Schleicher & Schuell) at 50 °C under non-stringent conditions. Percentage homology was calculated from the radioactivity of the hybrids relative to non-homologous and homologous controls using a Joyce-Loebl chromoscan 3 densitometer.

Maximum growth rate determination. The same initial starter culture (10%, v/v), grown at 50 °C, was used to inoculate all flasks. Growth (OD₆₀₀ versus time) was monitored at each temperature at least in triplicate and an aliquot of each culture was spread on a nutrient agar plate to check culture purity. All growth experiments were performed at a reciprocation rate of 300 r.p.m. Actual rates were determined using the formula $\ln x_t = \ln x_0 + \mu t$, where x_t = biomass concentration after time interval t h, x_0 = original biomass concentration, μ = specific growth rate (h⁻¹), t = time (h) and $\mu_{\max}$ = growth rate during exponential phase.

Preparation of whole-cell suspensions and cell-free extracts. Cultures were harvested by centrifugation at 6000 g for 15 min, then washed with 50 mM potassium phosphate buffer containing 1 mM dithiothreitol and 20% (v/v) glycerol (pH 7.2), and re-centrifuged for 15 min at 27000 g. The pellet was then resuspended in a minimal volume of 50 mM potassium phosphate buffer containing 1 mM dithiothreitol and 20% (v/v) glycerol (pH 7.2). Cell suspensions were sonicated in an ice-bath using an MSE Soniprep 150 (five bursts of 10 s duration, 10 µ amplitude, with 20 s intervals). Disrupted cells were centrifuged at 27000 g for 15 min and the supernatant was retained.

Protein determination. Protein concentrations were determined using the Bio-Rad Bradford protein determination kit with bovine serum albumin (fraction V; Sigma) as the protein standard.

Nitrile-degrading enzyme assay. Nitrile-degrading enzyme activity was assayed by measuring the production of ammonia by a modification of the phenol/hypochlorite method of Fawcett & Scott (1960). In determining nitrile hydratase activity by ammonia release, the specific activity of intracellular amidase was consistently found to be higher than that of the nitrile hydratase for the substrates tested. In light of these relative rates, we concluded that the nitrile to amide conversion was rate limiting and that measurement of ammonia released was a quantitative measurement of nitrile hydratase activity.

The following reagents were used for ammonia detection. Reagent A contained 0.59 M phenol and 1 mM sodium nitroprusside. Reagent B contained 110 mM sodium hypochlorite and 2 M sodium hydroxide. The standard assay was performed in duplicate at 50 °C in a reaction comprising, unless otherwise stated, 300- x µl 50 mM potassium phosphate buffer containing 1 mM dithiothreitol and 20% (v/v) glycerol (pH 7.2), x µl extract or whole-cell suspension and 5 µl 4.2 M acetonitrile or 1.2 M benzonitrile. Samples were incubated at 50 °C for 15 min. The cell-free extract reaction was quenched by addition of 100 µl of the assay mixture to 350 µl reagent B followed by rapid addition of 350 µl reagent A with vigorous mixing and incubation at 50 °C for 15 min. The whole-cell reaction was quenched by centrifugation at 2080 g for 3 min after incubation, followed by addition of 100 µl of the supernatant to 350 µl reagent B, followed by rapid addition of 350 µl reagent A with vigorous mixing and incubation at 50 °C for 15 min. The A₆₀₀ was then measured in a Cecil (model CE1020) spectrophotometer. One unit of acetonitrile- or

benzonitrile-degrading enzyme activity was defined as the amount of enzyme capable of releasing 1 μmol ammonia min^{-1} under standard reaction conditions (pH 7.2, 50 °C, 70 mM acetonitrile or 20 mM benzonitrile as substrate).

Temperature stability of enzyme activities. Aliquots of enzyme preparation, in 50 mM potassium phosphate buffer containing 1 mM dithiothreitol and 20% (v/v) glycerol (pH 7.2), were incubated at specified temperatures. At specific time intervals, samples were removed and placed on ice. Activity in all samples was subsequently determined using the standard enzyme assay procedure at 50 °C.

RESULTS AND DISCUSSION

Isolation strategies

Sediment samples collected from thermal sites in New Zealand (Tokaanu, Whakarewarewa, Waimangu and Taupo) were stored at -20 °C. Primary isolations were performed by spreading aqueous suspensions of these samples on minimal nutrient medium plates containing various nitriles as the sole carbon source. After incubation at 50 °C for 3–4 d, colonies were picked and re-streaked onto the same minimal nutrient medium plates. The plating cycle was repeated until pure cultures were obtained. Nitrile degradation was tentatively confirmed by incubating duplicate cultures on minimal nutrient medium plates supplemented and not supplemented with nitrile as the sole carbon source under otherwise identical conditions. The appearance of more rapid growth in the presence of the nitrile was taken as putative evidence for the presence of nitrile-degrading enzymes.

Nitrile-degrading isolates

Twenty-eight thermophilic isolates were examined for their ability to grow at 50 °C in liquid minimal nutrient media with nitriles (acetonitrile, benzonitrile, isovaleronitrile, propionitrile or acrylonitrile) as the sole carbon or nitrogen source. Growth on acetonitrile, propionitrile and isovaleronitrile gave higher growth rates and biomass yields than growth on benzonitrile or acrylonitrile. A single strain (designated Dac521) which showed higher nitrile-degrading enzyme activity during growth in liquid minimal nitrile media relative to other isolates was selected for further characterization.

Morphology and taxonomy

Isolate Dac521, a Gram-positive, sporulating, non-motile, rod-shaped organism ($2.0 \times 0.7 \mu\text{m}$) which grew between 35 and 70 °C on solid media and up to 73 °C in liquid media, was identified as a thermophilic *Bacillus* species using the API 50CH and 20E identification test strips (Logan & Berkeley, 1984). On nutrient agar plates, Dac521 colonies were of non-uniform size, circular form, convex elevation, with an entire margin and a yellow/grey colour. The organism was catalase- and oxidase-positive and fermented glucose, fructose and saccharose. Using DNA–DNA hybridization, strain Dac521 showed 87% homology to *B. pallidus* and less

Table 1. DNA hybridization results

Percentage DNA:DNA homology of ^{32}P -labelled Dac521 DNA against membrane-filter-bound test DNA. Percentage homology was calculated from the radioactivity of the hybrids relative to non-homologous and homologous controls. Data are mean values of quadruplicate experiments with standard deviations in parentheses.

Test species	Homology (%)
<i>B. thermoglucosidasius</i>	14 (4.8)
<i>B. thermodenitrificans</i>	15 (4.2)
<i>B. caldovelox</i>	2 (1.9)
<i>B. pallidus</i>	87 (17.7)
<i>B. stearothermophilus</i>	3 (2.4)
<i>B. caldolyticus</i>	2 (1.9)
<i>B. kaustophilus</i>	3 (2.0)
<i>B. smithii</i>	6 (3.5)
<i>B. thermocloacae</i>	4 (2.3)
<i>B. thermocatenuatus</i>	3 (3.2)
Isolate Dac521	100 (—)

than 15% homology to all other type strains tested (Table 1). According to White *et al.* (1993), this level of homology is strongly indicative of species designation and morphological and physiological characteristics were similar to those reported for the *B. pallidus* H14 type strain (White *et al.*, 1993; Scholz *et al.*, 1987).

Temperature optimum

The optimum growth temperature for *B. pallidus* Dac521 in nutrient broth was 50 °C (maximum growth rate, $\mu_{\text{max}} = 1.00 \pm 0.01 \text{ h}^{-1}$). μ_{max} fell to less than 10% of this value at 40 °C. No growth was detected at 74 °C or above. These results appear consistent with the description of *B. pallidus* Dac521 as a moderate thermophile. The μ_{max} of cultures grown in minimal nutrient media with acetonitrile or benzonitrile as the sole carbon and nitrogen source at 50 °C was $0.28 \pm 0.01 \text{ h}^{-1}$ and $0.06 \pm 0.01 \text{ h}^{-1}$, respectively, approximately 28% and 6% of the growth rates seen in nutrient broth at the same temperature.

Growth and activity profiles

B. pallidus Dac521 expressed aliphatic-nitrile- and amide-degrading activities when grown in nutrient broth (Fig. 1). Cell-free extracts from exponential and early stationary phase cultures degraded acetonitrile, propionitrile, acrylonitrile and the corresponding amides but no degradation of benzonitrile, isovaleronitrile or benzamide was detected. Nitrile-degrading enzyme activity was detectable from early exponential to mid stationary phase and the specific activity was highest at the transition from exponential to stationary phase. The specific activity was not enhanced by the addition of nitriles to the growth medium at the time of inoculation or at various times during culture growth (data not

Fig. 1. Growth on nutrient broth and acetonitrile-degrading enzyme activity profiles for *B. pallidus* Dac521. Culture growth was monitored by the OD₆₀₀ (□). At specific time-points, aliquots of the culture were removed, sonicated and acetonitrile-degrading activity and protein concentration were determined. Specific activity (■) is expressed as $\mu\text{mol ammonia produced min}^{-1} (\text{mg protein})^{-1}$ (U mg^{-1}).

shown). Under the culture conditions used, *B. pallidus* Dac521 had a short stationary phase followed by a rapid decrease in OD₆₀₀ with a concurrent decrease in detectable acetonitrile-degrading activity, consistent with a reduction or cessation of expression of the relevant gene(s) and induction of a specific degradation pathway (Moriwaka, 1974; Atalo & Gashe, 1993).

Under optimal conditions, *B. pallidus* Dac521 produced 1.3 U acetonitrile-degrading activity ($\text{mg dry wt cells}^{-1}$) compared to 0.1 U mg^{-1} for *Arthrobacter* sp. J-1 (Asano *et al.*, 1982).

Acetonitrile-degrading activity

DEAE-Sephacel ion-exchange chromatography (data not shown) of cell-free extracts of *B. pallidus* Dac521 grown in nutrient broth resulted in baseline separation of nitrile hydratase and amidase activities, capable of degradation of acetonitrile to acetamide and acetamide to acetic acid, respectively. As no other acetonitrile-degrading enzymes were detected in repeated chromatographic separations, we propose that acetonitrile degradation occurs via a hydratase–amidase pathway. The nitrile hydratase was shown to act on acetonitrile, propionitrile and acrylonitrile while the amidase hydrolysed the corresponding amides.

Microbial enzymes responsible for the catabolism of carbon compounds are often repressed by glucose and other hexoses (Toda, 1981; Tourneix *et al.*, 1986; Magasanik, 1961; Zimmermann & Scheel, 1977). Similarly, microbial enzymes intervening in nitrogen compound metabolism can be repressed by the ammonium ion (Yoch & Whiting, 1986). To investigate the possible occurrence of enzyme induction and/or repression, *B. pallidus* Dac521 was grown with a variety of carbon and nitrogen sources (Table 2).

Cell-free extracts of *B. pallidus* Dac521 grown in minimal nutrient media with succinate, pyruvate or

glucose as the sole carbon source and ammonium sulphate as the sole nitrogen source showed similar acetonitrile-degrading specific activities to that found in nutrient broth cultures, suggesting that catabolite repression or fixed nitrogen repression of aliphatic nitrile hydratase–amidase gene expression is not significant under the experimental conditions used. Other nitrile-degrading cultures (e.g. *Rhodococcus rhodochrous* J1; Nagasawa *et al.*, 1988) have also been shown to be insensitive to catabolite repression from carbon sources such as glucose and glycerol.

The acetonitrile-degrading activity of *B. pallidus* Dac521 was not enhanced by the addition of acetonitrile to nutrient broth or to minimal media with glucose or succinate as sole carbon source (Table 2). Conversely, when acetonitrile was supplied as the sole carbon and/or nitrogen source, the enzyme specific activities decreased to between 2% and 26% of those observed in nutrient broth. These results may be interpreted to imply that the aliphatic nitrile substrate suppresses expression of the acetonitrile-degrading enzyme gene, but may also suggest that expression is co-ordinately linked to cell growth parameters.

Aromatic-nitrile-degrading activity

When *B. pallidus* Dac521 was grown in minimal nutrient media with benzonitrile as the sole carbon and nitrogen source (Fig. 2), no acetonitrile-degrading activity was detectable but a distinct benzonitrile-degrading enzyme activity was observed. After a sequence of DEAE-Sephacel ion-exchange, phenyl-Sepharose hydrophobic interaction, and Superdex 200 and Superose 12 gel filtration chromatography steps, a single peak of benzonitrile-degrading activity was routinely eluted (data not shown). Using ammonia release assays, benzamide was not found to be a substrate over a wide range of concentrations (data not shown). Together these findings suggest strongly that benzonitrile degradation was via a nitrilase enzyme system.

Benzonitrilase specific activity increased during exponential phase but rapidly decreased during stationary phase, suggesting rapid 'switch-off' of benzonitrilase gene expression coupled with an active degradation mechanism. Maximum specific yields of benzonitrilase activity were approximately 0.2 U benzonitrilase activity (l culture^{-1}) compared to 5.0 U l^{-1} for *R. rhodochrous* J1 (Nagasawa *et al.*, 1988).

Although benzonitrile-degrading (benzonitrilase) activity was not detected until mid-late exponential growth phase (Fig. 2), acetonitrile degradation was not detected at any time during the growth phase. Furthermore, only benzonitrile-degrading activity was detectable in cultures grown in minimal media supplemented with both benzonitrile and acetonitrile (Table 2). These results are consistent with either total repression of 'acetonitrilase' gene expression (by benzonitrile) or quantitative inhibition of 'acetonitrilase' activity. However, when benzamide was substituted for benzonitrile in the

Table 2. Effect of carbon and nitrogen source on specific activities of acetonitrile-degrading enzymes

A 10% (v/v) inoculum of *B. pallidus* Dac521 grown in nutrient broth to an OD₆₀₀ of 1.2 was used to inoculate all flasks. *B. pallidus* Dac521 was grown in 50 ml vols minimal nutrient media in 250 ml flasks at 50 °C with a reciprocation rate of 300 r.p.m. Cultures were grown to early stationary phase before harvesting, sonication and determination of enzyme activity (acetonitrile/benzonitrile as substrate) and protein concentration. Data are mean values of triplicate or quadruplicate experiments $\pm$ standard errors. 0, No activity detected.

Carbon and nitrogen source	Acetonitrile degradation (U mg ⁻¹)	Benzonitrile degradation (U mg ⁻¹)
Nutrient broth	0.45 $\pm$ 0.02	0
20 mM succinate + 20 mM ammonium sulphate	0.40 $\pm$ 0.02	0
20 mM pyruvate + 20 mM ammonium sulphate	0.59 $\pm$ 0.17	0
20 mM glucose + 20 mM ammonium sulphate	0.42 $\pm$ 0.06	0
20 mM acetonitrile + 20 mM glucose	0.01 $\pm$ 0.001	0
20 mM acetonitrile + 20 mM succinate	0.05 $\pm$ 0.01	0
20 mM acetonitrile + 20 mM ammonium sulphate	0.12 $\pm$ 0.02	0
20 mM acetonitrile	0.08 $\pm$ 0.04	0
20 mM benzonitrile	0	0.13 $\pm$ 0.002
5 mM acetonitrile + 15 mM benzonitrile	0	0.15 $\pm$ 0.01
10 mM acetonitrile + 10 mM benzonitrile	0	0.18 $\pm$ 0.02
15 mM acetonitrile + 5 mM benzonitrile	0	0.10 $\pm$ 0.002

Fig. 2. Growth on minimal medium with benzonitrile as sole carbon source and benzonitrile-degrading enzyme activity profiles for *B. pallidus* Dac521. Culture growth was monitored by the OD₆₀₀ (□). At specific time-points, aliquots of the culture were removed, sonicated and benzonitrile-degrading activity and protein concentration were determined. Specific activity (■) is expressed as $\mu\text{mol ammonia produced min}^{-1} (\text{mg protein})^{-1}$ (U mg⁻¹).

growth medium under otherwise identical growth conditions, acetonitrile-degrading activity was detectable, suggesting that the aliphatic nitrile hydratase activity was suppressed, whether at the gene or enzyme level, by benzonitrile and not by the aromatic moiety alone. To further test the mode of 'inactivation', whole cells or cell-free extracts of *B. pallidus* Dac521 containing acetonitrile-degrading enzyme activity were incubated with 5 mM benzonitrile for 5 min at 50 °C. Subsequent assays failed to detect 'acetonitrilase' activity. Since 'acetonitrilase' activity was observed to be completely stable under similar conditions (50 °C, 5 min), we

conclude that inhibition of enzymic activity is the probable cause. Attempts to recover activity by centrifuging the benzonitrile-treated samples and resuspending the cells in fresh buffer containing no benzonitrile (each sample washed in triplicate) were unsuccessful (both positive and negative controls were as expected). Inhibition was therefore concluded to be effectively irreversible.

Similar patterns of enzyme activity, attributed to differential control of gene expression, have been reported previously for a number of mesophilic micro-organisms, including *Nocardia rhodochrous* (Collins & Knowles, 1983), *Arthrobacter* sp. strain J-1 (Asano *et al.*, 1982; Bandyopadhyay *et al.*, 1986), *R. rhodochrous* J1 (Kobayashi *et al.*, 1992) and *Fusarium solani* (Harper, 1977; Shimizu & Taguchi, 1969). However, both the results of our *in vivo* growth studies and *in vitro* inhibition assays strongly support the contention that the aliphatic-specific nitrile hydratase activity in *B. pallidus* Dac521 is inhibited rapidly and essentially irreversibly by benzonitrile.

Amidase expression

B. pallidus Dac521 appeared to express at least two distinct amidases. Growth in nutrient broth with benzonitrile or acetonitrile as the sole carbon and nitrogen source induced expression of an amidase which degraded acetamide but not benzamide. Conversely, growth with benzamide resulted in expression of a benzamide-specific amidase. In an analogous manner, *R. rhodochrous* J1 expresses two distinct amidases in a mutually exclusive and inducer-dependent fashion (Kobayashi *et al.*, 1993b).

Fig. 3. Temperature-activity profiles of *B. pallidus* Dac521 acetonitrile-degrading enzymes. The rate of acetonitrile (20 mM) degradation at a range of temperatures was determined in 50 mM potassium phosphate buffer containing 1 mM dithiothreitol and 20% (v/v) glycerol (pH 7.2). Whole-cell (●) and cell-free extract (○) specific activities are expressed as $\mu\text{mol ammonia produced min}^{-1} (\text{mg protein})^{-1}$ (U mg^{-1}).

Temperature-activity relationships

The temperature dependence of acetonitrile degradation by *B. pallidus* Dac521 whole-cell suspensions and cell-free extracts (Fig. 3) showed maximum rates at 55 and 50 °C, respectively. It is notable that the Q_{10} values for the two reaction systems are significantly different (estimated to be approximately 1.3 and 1.9, respectively).

Stability

The thermal stabilities of the acetonitrile- and benzonitrile-degrading enzyme systems were determined for both suspensions of whole cells and cell-free extracts

(Table 3). The greater apparent stability of both the acetonitrile- and benzonitrile-degrading enzyme systems in whole cells (compared with buffered cell-free extracts) may reflect either the release of compartmentalized degradation enzymes or a sensitivity to alterations in solution properties (pH, I , solutes, etc.). The substantial decrease in half-life between 50 and 60 °C (25-fold for cell-free extracts) for the acetonitrile-degrading activity suggests that this temperature range encompasses the putative T_m of one of the two enzymes involved in aliphatic nitrile degradation.

Biotransformations using mesophilic nitrile hydratases and nitrilases are typically carried out at low temperatures (0–10 °C) due to the instability of the enzymes (Hwang & Chang, 1989; Gilligan *et al.*, 1993). For example, *Brevibacterium* R312 and *R. rhodochrous* J1 nitrile hydratases were completely inactivated after a 10 min incubation at 30 °C (Nagasawa *et al.*, 1993). For purposes of comparison, assuming first order decay of activity to zero over 10 min, the nitrile hydratases of *Brevibacterium* R312 and *R. rhodochrous* J1 may have half-lives of 1–3 min at this temperature. In comparison, cell-free extracts of *B. pallidus* Dac521 had activity half-lives at 30 °C of 40 h and 2.5 d for the nitrile hydratase and benzonitrilase, respectively (Table 3). Thus we estimate that *B. pallidus* Dac521 nitrile hydratase and benzonitrilase are, respectively, approximately 1500-fold and 2500-fold more stable at 30 °C than the *Brevibacterium* R312 and *R. rhodochrous* J1 enzymes.

Immobilized *Brevibacterium* CH1 cells (used industrially for acrylamide production) had a half-life of activity of 7.5 h at 35 °C (Hwang & Chang, 1989). In comparison, non-immobilized *B. pallidus* Dac521 cells had activity half-lives of 5 d at 30 °C and 66.5 h at 40 °C.

Table 3. Half-lives of acetonitrile- and benzonitrile-degrading enzyme activity in whole-cell suspensions and cell-free extracts

Aliquots of enzyme, in 50 mM potassium phosphate buffer containing 1 mM dithiothreitol and 20% (v/v) glycerol (pH 7.2), were incubated at the specified temperatures. At specific time intervals, samples were removed and placed on ice. Activity in all samples was subsequently determined using the standard enzyme assay procedure at 50 °C. Data are mean values of triplicate experiments $\pm$ standard errors. ND, Not determined.

Temp. (°C)	Stability ($t_{1/2}$) of acetonitrile-degrading activity		Stability ($t_{1/2}$) of benzonitrile-degrading activity	
	Cell-free extract	Whole cells	Cell-free extract	Whole cells
4	ND	43.5 $\pm$ 0.5 d	ND	ND
22	ND	25.0 $\pm$ 0.0 d	ND	ND
30	40.0 $\pm$ 0.1 h	5.0 $\pm$ 0.2 d	2.5 $\pm$ 0.1 d	7.0 $\pm$ 0.1 d
40	15.0 $\pm$ 0.0 h	66.5 $\pm$ 0.5 h	1.0 $\pm$ 0.1 d	3.5 $\pm$ 0.1 d
50	3.1 $\pm$ 0.1 h	4.5 $\pm$ 0.0 h	3.8 $\pm$ 0.2 h	6.5 $\pm$ 0.2 h
55	1.9 $\pm$ 0.1 h	ND	ND	ND
60	7.3 $\pm$ 0.1 min	8.2 $\pm$ 0.1 min	14.9 $\pm$ 1.1 min	34.0 $\pm$ 2.0 min
70	ND	ND	< 1.0 min	< 1.0 min

On the basis of thermal stability alone, these results suggest that the *B. pallidus* Dac521 nitrile hydratase and nitrilase may have significant industrial advantages over their mesophilic counterparts.

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