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Metabolism of Benzonitrile and Butyronitrile by *Klebsiella pneumoniae*

MOHAMED S. NAWAZ, THOMAS M. HEINZE, AND CARL E. CERNIGLIA*

National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas 72079

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A strain of *Klebsiella pneumoniae* that used aliphatic nitriles as the sole source of nitrogen was adapted to benzonitrile as the sole source of carbon and nitrogen. Gas chromatographic and mass spectral analyses of culture filtrates indicated that *K. pneumoniae* metabolized 8.4 mM benzonitrile to 4.0 mM benzoic acid and 2.7 mM ammonia. In addition, butyronitrile was metabolized to butyramide and ammonia. The isolate also degraded mixtures of benzonitrile and aliphatic nitriles. Cell extracts contained nitrile hydratase and amidase activities. The enzyme activities were higher with butyronitrile and butyramide than with benzonitrile and benzamide, and amidase activities were twofold higher than nitrile hydratase activities. *K. pneumoniae* appears promising for the bioremediation of sites contaminated with aliphatic and aromatic nitriles.

Biodegradation of nitriles is of considerable importance because of their deleterious health effects (1, 22). The microbial metabolism of nitriles proceeds through two different pathways. Nitrilase (EC 3.5.5.1) participates in the direct conversion of nitriles to their carboxylic acids and ammonia (12, 18). Asano et al. (2) proposed a second pathway involving a nitrile hydratase (EC 4.2.1.84) that mediates the conversion of nitriles to their amides. An amidase (EC 3.5.1.4) converts the amides to their corresponding carboxylic acids and ammonia (3). Microorganisms that contain only nitrilase fail to metabolize amides (4, 12).

Numerous microorganisms that utilize aliphatic nitriles have been reported (2, 3, 5, 6, 21, 23). These organisms, which contain a nitrile hydratase and an amidase to metabolize nitriles, rarely degrade aromatic nitriles or their amides. A few microorganisms metabolize benzonitrile, an active ingredient of some widely used herbicides such as dichlobenil (2,6-dichlorobenzonitrile), bromoxynil (3,5-dibromo-4-hydroxybenzonitrile), and ioxynil (4-hydroxy-3,5-diiodobenzonitrile). Harper isolated strains of *Rhodococcus rhodochrous* (7, 9) and *Fusarium solani* (8) that utilized benzonitrile as the sole source of carbon and nitrogen. Both microorganisms contained a nitrilase that hydrolyzed benzonitrile to benzoic acid and ammonia. *Arthrobacter* sp. strain J-1 (4), which metabolizes aliphatic nitriles (24), has also been found to utilize benzonitrile as a growth substrate (4). This strain contains two different nitrilases that convert benzonitrile to benzoic acid and ammonia. However, this isolate lost its ability to metabolize aliphatic nitriles after adaptation on benzonitrile (4). A strain of *Klebsiella pneumoniae* subsp. *ozaenae* that degraded bromoxynil failed to degrade benzonitrile (15).

In this study we provide the first evidence for a strain of *K. pneumoniae* that uses benzonitrile as the sole source of carbon and nitrogen. This strain also metabolized butyronitrile and other aliphatic nitriles as nitrogen sources. In addition, *K. pneumoniae* simultaneously used benzonitrile as the sole source of carbon and nitrogen and several aliphatic nitriles as supplementary sources of nitrogen.

* Corresponding author.

MATERIALS AND METHODS

Chemicals. Aldrich Chemical Co. (Milwaukee, Wis.) was the supplier of all nitriles and amides used in this study. The purity of these compounds was >99.0%. High-performance liquid chromatography-grade methylene chloride and ethyl acetate were purchased from J. T. Baker Chemical Co. (Phillipsburg, N.J.). All other chemicals were of analytical grade and available commercially.

Microorganism and culture conditions. An isolate of *K. pneumoniae* NCTR 1 that utilized acrylonitrile as the sole source of nitrogen (20) was cultured on phosphate buffer medium (PBM) supplemented with 8.4 mM benzonitrile as the sole source of carbon and nitrogen. One milliliter of a 48-h bacterial suspension (A_{600} = 1.0) was used as the inoculum for 40 ml of PBM. The protein content was 820 μg/ml of inoculum.

Metabolism of benzonitrile and butyronitrile by *K. pneumoniae*. The bacterium was cultured in a 2-liter Fernbach flask containing 500 ml of PBM supplemented with 8.4 mM benzonitrile. Samples (50 ml) were removed at 24-h intervals from 0 to 96 h. After determination of bacterial growth and ammonia, indicating cyanide cleavage (19), the bacterial cells were centrifuged at 10,000 × *g* for 15 min. The supernatant was divided into two portions of 25 ml each. The pH of one portion was adjusted to 12.0 with 6 M NaOH, and the pH of the other portion was acidified to 2.0 with concentrated HCl. The supernatants were extracted with 75 ml of ethyl acetate. The ethyl acetate fractions were pooled, dried, and concentrated under vacuum at 30°C. The residues were dissolved in 2 ml of ethyl acetate before analysis by gas chromatography (GC). Benzonitrile metabolites were further confirmed by GC-mass spectrometry (MS).

The metabolism of butyronitrile was determined by culturing the bacterium in 125-ml Pyrex flasks containing 40 ml of PBM supplemented with 36 mM butyronitrile and 0.1 g of glucose per liter. Samples (3 ml) were removed at 24-h intervals, and the bacterial growth was determined. The bacterial cells were harvested by centrifugation, and the ammonia concentration of the supernatant was determined. Methylene chloride extracts of the supernatant were analyzed for metabolites by GC and GC-MS.

Enzyme assays. Cell extracts prepared as described previously (20) served as the crude enzyme source. Nitrile

hydratase and amidase activities were assayed by measuring the ammonia released from the substrates (2, 3).

Analytical methods. Bacterial growth was determined by measuring the A_{600} with a DU-7 spectrophotometer (Beckman Instruments, Fullerton, Calif.) against a blank of PBM. The protein content of the cell extract was determined by the method of Lowry et al. (13). Ammonia was determined colorimetrically (10).

Nitriles and their metabolites were detected and quantified by GS, and the metabolites were confirmed by GC-MS (20).

RESULTS

Growth of *K. pneumoniae* on aromatic nitriles and related compounds. Benzonitrile and 4-cyanopyridine were the only aromatic nitriles to support the growth of the bacterium. Benzamide, an amide of benzonitrile when supplied as the sole source of carbon and nitrogen, supported higher bacterial growth and ammonia accumulation than did any other substrate tested. Similarly, the isolate also extensively utilized butyramide as the sole source of carbon and nitrogen. Benzoic acid and butyric acid, which are carboxylic acid intermediates of benzonitrile and butyronitrile, respectively, supported growth of the bacterium as the sole source of carbon (data not shown).

Metabolism of benzonitrile and butyronitrile by *K. pneumoniae*. Preliminary studies indicated that *K. pneumoniae* metabolized 8.4 mM benzonitrile and 36 mM butyronitrile during 72 and 96 h of incubation, respectively. GC analysis of the acidified and concentrated broth indicated that benzonitrile was transformed into a compound with a retention time of 14.4 min (Fig. 1A). This compound was tentatively identified as benzoic acid by comparing its retention time with that of an authentic standard. GC-MS analysis of the 72-h culture filtrate confirmed the presence of benzoate. The mass spectrum had prominent ions at m/z 122 (M^+), 105 (M^+-OH), 77 (M^+-CO_2H), and 51 (M^+-$C_2H_3O_2$). GC analysis failed to detect the presence of metabolites in neutral or alkaline concentrates.

Similarly, the metabolites of butyronitrile were initially detected by measuring GC retention times. Analysis of a methylene chloride extract collected after 24 h indicated the partial transformation of butyronitrile to a compound with a retention time of 8.8 min (Fig. 1B). This compound was identified as butyramide by comparing its retention time with that of an authentic standard. GC-MS analysis of the 96-h culture filtrate confirmed the presence of butyramide. The mass spectrum had prominent ions at m/z 87 (M^+), 72 (M^+-CH_3), 71 (M^+-NH_2), 59 (M^+-C_2H_4), 44 (M^+-C_3H_7), and 43 (M^+-$CONH_2$).

The kinetics of benzonitrile metabolism indicated that the maximum accumulations of benzoate and ammonia were 4.0 and 2.7 mM, respectively, at 48 h (Fig. 2). Similar studies with butyronitrile indicated that the maximum accumulations of butyramide and ammonia were 15 and 9.6 mM, respectively, after 48 h (Fig. 3). Butyric acid, a carboxylic acid intermediate of butyronitrile metabolism, was not detected in the culture medium at any time in the growth cycle.

Cometabolism of benzonitrile and butyronitrile and related compounds. The bacterium completely degraded a mixture of 8.4 mM benzonitrile and 4.5 mM butyronitrile as the sole sources of carbon and nitrogen (Table 1). However, higher concentrations of butyronitrile inhibited benzonitrile utilization.

Similarly, the bacterium metabolized 4.5 mM of other aliphatic nitriles in the presence of benzonitrile (Table 1).

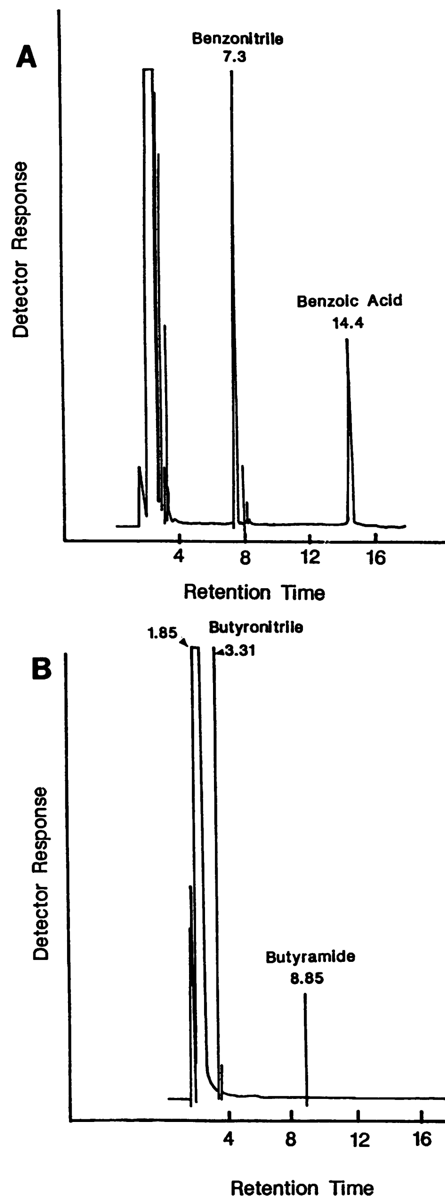

FIG. 1. Detection and identification of benzonitrile and butyronitrile metabolites produced by *K. pneumoniae*. (A) GC profile of acidified concentrated broth recovered after 24 h of incubation. Benzonitrile and benzoate had retention times of 7.3 and 14.4 min, respectively. (B) GC profile of butyronitrile after 24 h of incubation. Butyronitrile and butyramide had retention times of 3.3 and 8.8 min, respectively.

However, benzonitrile supplemented with butyronitrile, propionitrile, and glutaronitrile supported higher biomass and ammonia accumulation than did other mixtures. No significant change in benzoate content was observed between control cultures and cultures supplied with mixed substrate.

Enzyme assays. Nitrile hydratase and amidase activities were linear with time and enzyme and substrate concentrations. The optimal aliphatic nitrile hydratase and amidase activities were obtained with higher concentrations of substrates (10 mM). However, the optimal aromatic nitrile hydratase and amidase activities were obtained with lower substrate concentrations (1 mM). The optimal temperature

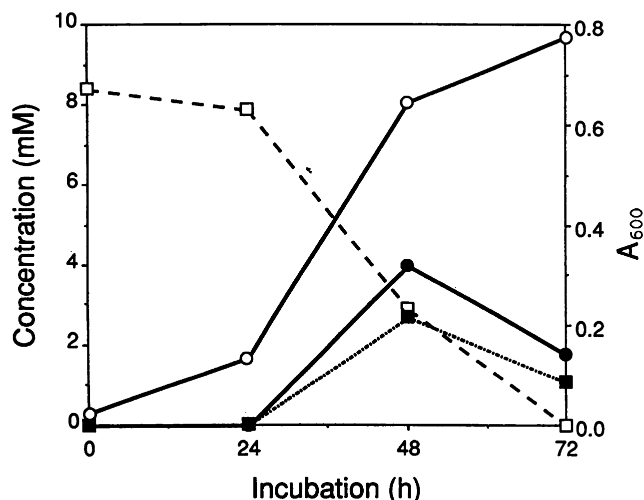

FIG. 2. Metabolism of benzonitrile by *K. pneumoniae*. Cultivation was carried out at 28°C in 500 ml of PBM containing 8.4 mM of benzonitrile. Symbols: □, benzonitrile; ●, benzoic acid; ■, ammonia; ○, A_{600}.

for both enzymes was approximately 30°C, and the enzyme activities were maximum at pH 8.0. Very little enzyme activity occurred below pH 5.5 or at higher temperatures.

The maximum enzyme activity of the isolate occurred in cells harvested after 48 h of incubation. The amidase activities with both aliphatic and aromatic amides were higher than the nitrile hydratase activity (Fig. 4). The enzymes were more active on aliphatic butyronitrile and its amide than on aromatic benzonitrile and its amide.

DISCUSSION

Our earlier study (20) indicated that *K. pneumoniae* utilized benzonitrile and other aliphatic nitriles as nitrogen sources. After three subcultures at 48-h intervals on benzonitrile as the sole growth substrate, the strain utilized benzonitrile as the sole source of carbon and nitrogen (Fig. 2). However, the adapted strain, like the unadapted isolate,

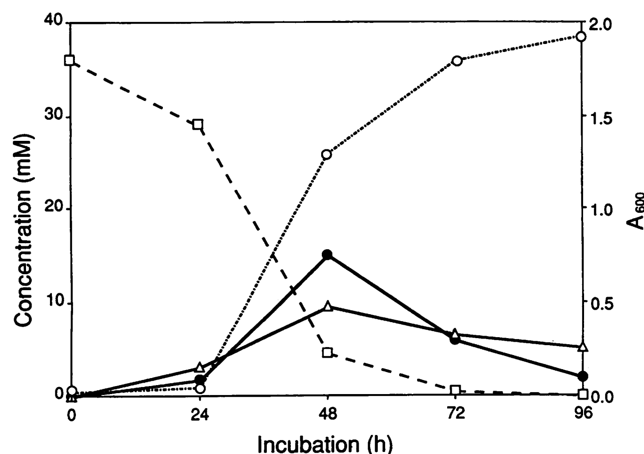

FIG. 3. Metabolism of butyronitrile by *K. pneumoniae*. Cultivation was carried out at 28°C in 500 ml of PBM containing 36 mM butyronitrile. Symbols: □, butyronitrile; ●, butyramide; △, ammonia; ○, A_{600}.

TABLE 1. Simultaneous metabolism of benzonitrile and various aliphatic nitriles[a]

Growth substrate(s)	A_{600}	Ammonia (mM)
Benzonitrile + butyronitrile	1.100	1.6
Benzonitrile + acetonitrile	0.801	1.7
Benzonitrile + glutaronitrile	0.831	3.3
Benzonitrile + propionitrile	0.957	4.3
Benzonitrile + succinonitrile	0.793	3.0
Benzonitrile + methacrylonitrile	0.805	3.5
Benzonitrile (control)	0.770	1.1

[a] The bacterial isolate was cultured in a 125-ml Pyrex flask containing 40 ml of PBM and supplemented with 8.4 mM benzonitrile as the sole source of carbon and nitrogen. Aliphatic nitriles (4.5 mM) were added as additional growth substrates. Bacterial growth and ammonia accumulation were determined after 96 h. The initial A_{600} was 0.03.

needed glucose supplementation to degrade butyronitrile, an aliphatic nitrile extensively used as an industrial solvent (Fig. 3). In addition, the adapted strain utilized a mixture of benzonitrile and other aliphatic nitriles (Table 1). Degradation of aliphatic nitriles in the presence of benzonitrile proceeded without glucose supplementation. To our knowledge, this is the first report of a microorganism that simultaneously metabolizes of both aliphatic and aromatic nitriles.

DiGeronimo and Antoine (6) isolated a *R. rhodochrous* (*Nocardia rhodochrous*) strain that metabolized acetonitrile to acetamide, acetic acid, and ammonia. The same organism degraded propionitrile to propionic acid and ammonia, but propionamide was not detected in the culture medium. Similarly, Nawaz et al. (19) reported that *Pseudomonas putida* degraded acetonitrile to acetic acid and ammonia. No

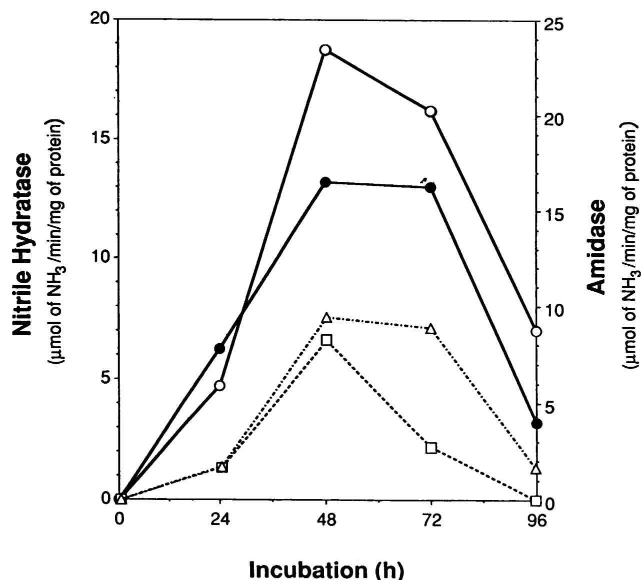

FIG. 4. Nitrile hydratase and amidase activities during cultivation of *K. pneumoniae*. Cultivation was carried out at 28°C in a 2-liter Fernbach flask with 500 ml of PBM containing 8.4 mM benzonitrile or 36 mM butyronitrile as the growth substrate. Enzyme activities were assayed by measuring the concentration of ammonia. Symbols: □, nitrile hydratase activity with benzonitrile as the substrate; ●, nitrile hydratase activity with butyronitrile as the substrate; △, amidase activity with benzamide as the substrate; ○, amidase activity with butyramide as the substrate.

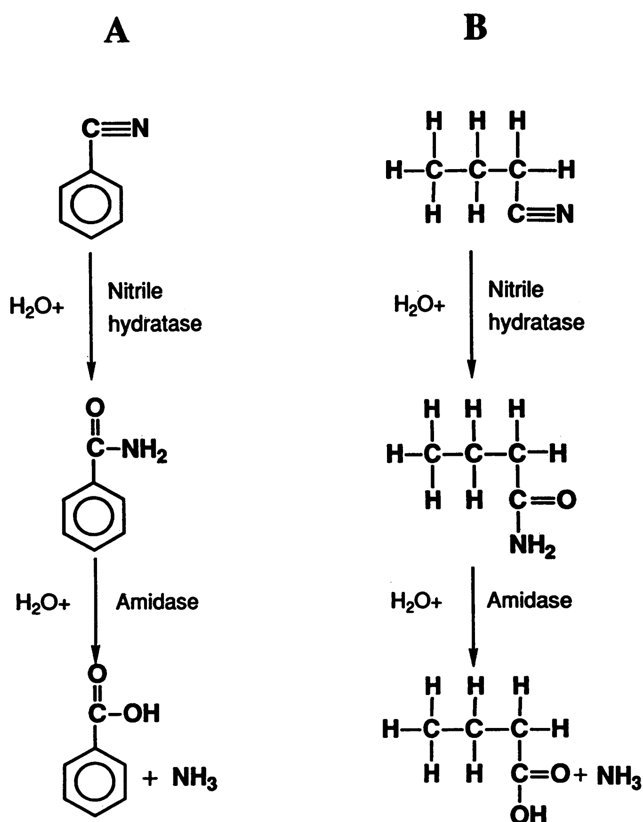

FIG. 5. Proposed pathways of benzonitrile (A) and butyronitrile (B) metabolism by *K. pneumoniae*.

acetamide was detected in the culture medium. Both bacterial isolates extensively utilized propionamide and acetamide as the sole sources of carbon and nitrogen. Based on these observations, these investigators concluded that they failed to detect the respective amide because it was rapidly utilized. Earlier, Mimura et al. (16) had reported the detection of acetamide and ammonia as a result of acetonitrile degradation by a *Corynebacterium* sp. Although acetic acid was not detected in the growth medium, the investigators assumed it to be an intermediate of acetonitrile metabolism because of the presence of ammonia.

In our study, *K. pneumoniae* rapidly metabolized benzonitrile to benzoic acid and ammonia (Fig. 2). Butyronitrile was metabolized to butyramide and ammonia (Fig. 3). Furthermore, the isolate readily metabolized benzamide, butyramide, benzoic acid, and butyric acid. Although we did not detect benzamide and butyric acid as a result of benzonitrile and butyronitrile metabolism, we suggest that these compounds may be intermediates of benzonitrile and butyronitrile metabolism. Our failure to detect them may be attributed to the rapid utilization of these intermediates by the bacterium as additional growth substrates.

Nitrilases of *Fusarium* sp. (8), *Rhodococcus* sp. (9), and *Arthrobacter* sp. (4) are responsible for the metabolism of benzonitrile. None of these nitrilases catabolizes aromatic amides or aliphatic nitriles. In this study, we demonstrated an alternate degradative pathway (Fig. 5) that involves a nitrile hydratase and an amidase (Fig. 4) responsible for the metabolism of benzonitrile. Similar enzymes were also responsible for the metabolism of butyronitrile (Fig. 4). Although aliphatic butyronitrile and its amide were highly preferable to the aromatic benzonitrile and its amide as enzyme substrates, the enzyme activities were lower than the activities of the unadapted strain (20); contrary to our earlier observation (20), the amidase activity of the adapted strain was higher than its nitrile hydratase activity (Fig. 4).

Nitrile-degrading enzymes have been commercially exploited for the industrial production of amides (18, 23) and organic acids (11, 12, 14, 17). Although numerous microorganisms have been reported to convert aliphatic nitriles to amides or acids, only a few microorganisms act on aromatic nitriles or their amides. The *K. pneumoniae* described in this investigation has the advantage of metabolizing aliphatic nitriles to their amides. In addition, the organism can convert aromatic nitriles and their amides to aromatic acids. These unique duel enzymatic characteristics of the bacterium may be useful for the industrial production of higher-value aliphatic amides and aromatic acids. Furthermore, the ability of the organism to simultaneously degrade mixtures of aromatic and aliphatic nitriles may be useful for the detoxification or bioremediation of sites contaminated with synthetic nitriles. Investigations on the synthesis of aliphatic amides or aromatic acids by purified nitrile-degrading enzymes are in progress in this laboratory.

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