Microbial Isobutyronitrile Utilization under Haloalkaline Conditions

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The utilization of isobutyronitrile (iBN) as a C and N source under haloalkaline conditions by microbial communities from soda lake sediments and soda soils was studied. In both cases, a consortium consisting of two different bacterial species capable of the complete degradation and utilization of iBN at pH 10 was selected. The soda lake sediment consortium consisted of a new actinobacterium and a gammmaproteobacterium from the genus Marinospirillum. The former was capable of fast hydrolysis of aliphatic nitriles to the corresponding amides and much-slower further hydrolysis of the amides to carboxylic acids. Its partner cannot hydrolyze nitriles but grew rapidly on amides and carboxylic acids, thus acting as a scavenger of products released by the actinobacterium. The soda soil consortium consisted of two Bacillus species (RNA group 1). One of them initiated nitrile hydrolysis, and the other utilized the hydrolysis products isobutyroamide (iBA) and isobutyrate (iB). In contrast to the actinobacterium, the nitrile-hydrolyzing soil Bacillus grew rapidly with hydrolysis products, but it was dependent on vitamins most probably supplied by its product-utilizing partner. All four bacterial strains isolated were moderately salt-tolerant alkaliphiles with a pH range for growth from pH 7.0 to 8.5 up to 10.3 to 10.5. However, both their nitrile hydratase and amidase activities had a near-neutral pH optimum, indicating an intracellular localization of these enzymes. Despite this fact, the study demonstrated a possibility of whole-cell biocatalytic hydrolysis of various nitriles at haloalkaline conditions.

Nitriles are organic compounds containing a —C=N (nitrile) bond. They are mostly industrially produced, as intermediates and building blocks in organic synthesis and as organic solvents, but there are also a few examples of naturally occurring nitriles, formed by cyanogenic plants from cyanide (23). In addition, simple aliphatic nitriles, such as isobutyronitrile (iBN), can be produced during the anaerobic degradation of amino acids (7).

Most of the nitriles are hydrophobic, toxic compounds that are difficult to degrade. Therefore, the environmental role of the enzymatic conversion of nitriles is very important. Basically, two different enzymatic mechanisms resulting in the conversion of nitriles to their corresponding carboxylic acids are known. The metalloenzyme nitrile hydratase hydrolyzes a wide range of aliphatic, arylnaphatic, and aromatic nitriles to their corresponding amides (R-CONH2), which can be further converted into carboxylic acids and ammonium by amidases. The organisms producing nitrile hydratases usually also produce amidases (10). In the case of an organism with a weak amidase activity, an association with an amide-specializing partner can be very efficient in complete nitrile biodegradation (12). Another enzyme family of nitrilases performs a single-step hydrolysis of nitriles, mostly aromatic, into acids and ammonium, although weak production of amides as by-products was reported in some cases (9, 11, 17). The microorganisms possessing these enzymes are valuable biocatalysts and can be used either in (enantioselective) organic synthesis or in environmental biotechnology (1, 2, 6, 12, 14). This stimulated the search for active producers of nitrile-hydrolyzing enzymes (13), as well as the screening of environmental DNA and whole-genome sequences for the genes encoding new nitrile-degrading enzymes (23). Currently, many strains, mostly bacterial, but also several fungal, are known as active producers of nitrile-hydrolyzing enzymes. The best-studied group among them, producing extremely active nitrile hydratases and nitrilases, belongs to the genus Rhodococcus in the actinobacteria (3, 10). So far, all known nitrile-degrading microorganisms are neutrophilic, i.e., they grow optimally at neutral pH values.

Soda lakes and soda solonchak soils are naturally occurring saline habitats with a constant high pH of around 10 due to the high alkaline-buffering capacity of dissolved sodium carbonates. These habitats harbor mostly haloalkaliphilic prokaryotic microbial communities (8, 20, 25). Recently, we have described the first example of a bacterium, Natronocella acetinitrilica, isolated from soda lake sediments, capable of growth with acetate- and propionitrile as carbon, energy, and nitrogen sources under haloalkaline conditions (21). Enzymatic nitrile hydrolysis at highly alkaline conditions might have certain advantages, particularly when cyanide is involved in the reaction process. For example, the well-known Strecker reaction could be coupled with enzymatic α-aminonitrile hydrolysis to (enantioselectively) produce α-αminoadamides and α-amino acids (5).

In this paper, the possibility of the degradation of more-complex nitrile molecules by haloalkaliphilic bacteria from soda habitats is described. The results demonstrated that iBN can be efficiently utilized as a carbon, energy, and nitrogen source at a high pH by the concerted action of at least two different bacterial species producing nitrile hydratase and amidase (Fig. 1).

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MATERIALS AND METHODS

Sediment and soil samples. A sediment mixture made from 10 samples from Kulunda Steppe (southwestern Siberia, Altai, Russia) soda lakes and a mixture of 20 samples of solonchak soda soils (Kulunda Steppe and northeastern Mongolia) were used as the inocula to be enriched for culturing iBN-degrading haloalkaliphiles. The pHs, alkalinites, and total salinities of the lakes were within the ranges of 9.5 to 11.05, 0.2 to 4.0 M, and 50 to 400 g liter\(^{-1}\), respectively (20). The pHs of water extracts from the solonchak soil samples varied from 9.5 to 10.5, the total alkalinity from 0.05 to 1.2 M, and the total salt content from 30 to 200 g kg\(^{-1}\). The dominant ions in both habitats were Na\(^+\), Mg\(^{2+}\), HCO\(_3^-\), \(\text{CO}_3^{2-}\), Cl\(^-\), and SO\(_4^{2-}\).

Media compositions and growth conditions. A mineral medium based on sodium carbonate buffer at pH 10 and 0.6 M total Na\(^+\), containing 22 g liter\(^{-1}\) Na\(_2\)CO\(_3\), 8 g liter\(^{-1}\) NaHCO\(_3\), 6 g liter\(^{-1}\) NaCl, 0.5 g liter\(^{-1}\) KH\(_2\)PO\(_4\), was used for the enrichment and pure culture experiments. The pH of this medium was stable even after prolonged incubation. After being sterilized, the medium was supplemented with 1 ml liter\(^{-1}\) trace metal solution (16), 1 mM MgSO\(_4\), and 0.1 mg liter\(^{-1}\) of filter-sterilized vitamin B\(_12\). Enrichment was performed in 100-ml serum bottles closed with rubber septa (to prevent substrate loss) containing 20 ml medium and 1 ml sediment or 1 g soil. In the case of nitriles and their corresponding amides, these compounds were used as both C and N sources, while in the case of carboxylic acids, the medium was supplemented with 4 mM NH\(_4\)Cl. iBN to a final concentration of 2 to 10 mM was added directly to each culture vessel before the vessel was closed. In the case of the solid medium (the solidifying agent was Noble agar; Difco), iBN was added after the medium was cooled down to 50°C, to prevent excessive loss of substrate. Liquid cultures were incubated on a rotary shaker at 100 rpm and 30°C and were periodically checked for ammonia production. When the ammonia concentration reached 2 mM, the culture was transferred into a new medium at a 1:100 dilution. After 3 to 4 successful 1:100 transfers, the culture was serially diluted up to 10\(^{-11}\). The culture from a maximal positive dilution was plated onto solid medium, either by surface spreading or by the agar-shake technique. The plates were incubated in closed jars for 30 days. Separate colonies were placed into 5 ml liquid medium with iBN or isobutyroamide (iBA) in 30-ml serum bottles closed with rubber septa. Positive cultures were plated again to check for purity.

Growth experiments with pure cultures were performed in 250-ml closed serum bottles with 50 ml liquid on a rotary shaker at 100 to 150 rpm and 30°C. Substrates were used at a 5 to 20 mM concentration. The growth was monitored by assessing the optical density, and the degradation of nitriles was followed by assessing the level of ammonium production, the disappearance of the substrate, and the formation of intermediates. The pH profiling of the growth of the cultures was done in the medium containing 0.6 M total Na\(^+\) either as NaCl (pH 6.5 to 8.0) or NaHCO\(_3\)/Na\(_2\)CO\(_3\) (pH 8.5 to 11.0) (19). The salt dependence for the growth of the cultures was investigated in a range of sodium carbonate-based media containing 0.1 to 3.0 M total Na\(^+\) at pH 10.

FIG. 1. Scheme of iBN hydrolysis by a nitrile hydratase (NHas)/amidase system through iBA to iBN.

centrification of the denatured protein, the supernatant was either injected directly into a high-pressure liquid chromatography column or diluted first with MilliQ water when necessary.

Analytical procedures. The protein concentration was measured by the Lowry method. The ammonium concentration was determined by the phenol-hypochlorite method according to Weatherburn (24). Nitriles, amides, and carboxylic acids were detected by high-pressure liquid chromatography. All compounds were detected by using a Merck Chromolith SpeedROD RP-18e column (50 \(\times\) 4.6 mm), except for acetonitrile, acetamide, and acetic acid. Acrylonitrile, methacrylonitrile, butyronitrile, iBN, valeronitrile, nicotinonitrile, and their corresponding amides and acids were separated by using an eluent of MilliQ water (98.9%), acetonitrile (1%), and acetic acid (0.1%). Benzonitrile, phenylacetonitrile, capronitrile, and their corresponding amides and acids were separated by using an eluent of MilliQ water (89.9%), acetonitrile (10%), and acetic acid (0.1%). Propiononitrile and its corresponding amide and acid were separated by using an eluent of MilliQ water (99.9%) and acetic acid (0.1%). All separations on the SpeedROD were carried out at 21°C and with a flow rate of 1 ml/min. Acetonitrile, capronitrile, and acetic acid were detected by using a Phenomenex Rexx ROA-aromatic acid H\(^+\) column (300 \(\times\) 8.0 mm; 8 microns) with an eluent consisting of MilliQ water with 0.1 M trifluoroacetic acid (0.6 ml/min) and a column temperature of 60°C. The aliphatic nitriles were detected by using a Shimadzu RID 10A refractive index detector. A wavelength of 210 nm was used to detect all aliphatic amides and acids, and a wavelength of 250 nm was used to detect all aromatic amides and acids.

Phase-contrast microphotographs were obtained by using a Zeiss Axiosplan Imaging 2 microscope (Göttingen, Germany). For electron microscopy, cells were fixed with glutaraldehyde (final concentration 3%, vol/vol) and stained with 1% (wt/vol) uranyl acetate for positive contrast. For thin sectioning, the cells were fixed in 1% (wt/vol) OsO\(_4\) and 0.5 M NaCl for 3 h at room temperature, washed, stained overnight with 1% (wt/vol) uranyl acetate, dehydrated in an ethanol series, and embedded in Epon resin. The thin sections were stained with 1% (wt/vol) lead citrate. The isolation of the DNA and subsequent determination of the G + C content and the DNA-DNA hybridization were performed by using the thermal denaturation/reassociation technique (4, 15).

Genomic DNA for phylogenetic analysis was extracted from the cells by using an UltraClean soil DNA extraction kit (MoBio Laboratories, United States) following the manufacturer’s instructions. The 16S rRNA genes were amplified using general bacterial primers. The PCR products were purified by low-melting agarose by using a Wizard PCR Prep kit (Promega, United States) according to the manufacturer’s instructions. Sequencing was performed by using a BigDye Terminator v.3.1 sequencing reaction kit on an ABI 3730 DNA automatic sequencer (Applied Biosystems, Inc., United States). The sequences were first compared with those stored in GenBank by using the BLAST algorithm. The sequences were aligned with those from GenBank by using ClustalW. Phylogenetic trees were constructed with four different algorithms using the TREECONW software package (22).

Nucleotide sequence accession numbers. The GenBank accession numbers for the 16S rRNA gene sequences of the strains ANL-iso2, ANL-iso4, ANL-iso6, and ANL-iso2 are EF422408, EF422411, EF422409, and EF422410, respectively.

RESULTS

Soda lake sediment coculture. In a soda lake sediment primary enrichment culture, iBN degradation was noticeable after 2 weeks of incubation. Several stabilizing 1:100 transfers resulted in a persistent coculture which could not be separated by the serial dilution technique. It consisted of long, motile spirilla as a dominant morphotype and short, nonmotile rods as a minor component. Since colony formation was not observed on solid medium with iBN, attempts were made to separate the
consortium by dilution to extinction under different conditions. When the iBN concentration was gradually increased from 2 to 10 mM, the spirilla were markedly inhibited, which eventually allowed the purification of the iBN-degrading rod-shaped component, designated strain ANL-iso2 (NCCB 100119), in a pure culture. In a pure culture, ANL-iso2 was able to form flat, spreading microcolonies on iBN agar after a month of incubation. When iBN was replaced by iBA or isobutyrate (iB), the spirilla grew much faster, and they also formed colonies on iBA agar, allowing the isolation of a pure culture designated strain ANL-isoa. This bacterium was not able to utilize iBN. 16S rRNA gene sequence analysis showed that the nitrile-degrading strain ANL-iso2 represents a new lineage in the *Actinobacteria*, mostly consisting of uncultured representatives, with maximum sequence similarities of 94 to 95% to clones from the soda lake Mono Lake. Its partner, strain ANL-isoa, is a new species within the genus *Marinospirillum* in the *Gamma-proteobacteria*, with 95% sequence similarity to its closest relative, *M. alkaliphilum*.

The actinobacterium ANL-iso2 grew with propionitrile (C₃), butyronitrile (C₄), valeronitrile (C₅), and capronitrile (C₆), in addition to iBN, as carbon, energy, and nitrogen sources (Table 1), with two phases of growth clearly distinguished. The first, rapid phase of nitrile hydrolysis to the corresponding amide, with little biomass growth, was followed by the second, much longer phase of biomass growth with amide/acid utilization (Fig. 2a). Clearly, the utilization of hydrolysis products was the bottleneck for complete nitrile metabolism in this bacterium. Its partner, *Marinospirillum* ANL-isoa, was unable to utilize nitriles, but grew at least 3 to 6 times faster than the nitrile-degrading organism on the products of nitrile hydrolysis and other simple compounds (Table 1).

**Soda soil coculture**. The soda soil enrichment culture developed much faster than the soda lake sediment enrichment culture, consuming 4 mM iBN within a week. Despite the development of quite a dense culture, serial dilutions of the 4 mM iBN medium that still contained at least two different spore-forming cell phenotypes were only positive up to a 10⁻³ dilution. The phenotype with longer cells was separated from the coculture on solid medium with iBA, and the strain was designated ANL-isoa2. It was not able to grow with iBN, however. The second organism, apparently the iBN degrader, was difficult to isolate, since it was obligately dependent on its partner, in contrast to the iBN-degrading actinobacterium. Eventually, colony formation with iBN was observed, using the agar-shake technique, in somewhat unusual fashion. Instead of a homogenous distribution, patches of growth were formed, each patch consisting of numerous small colonies (belonging to strain ANL-isoa2) clustered around one or two much bigger central colonies. The latter belonged to the iBN-degrading organism, as was proven by transferring those colonies into liquid medium with iBN. It was designated strain ANL-iso4 (NCCB 100120). It grew relatively fast with iBN, iBA, and iB as carbon and energy sources, but only in the presence of yeast extract (50 mg liter⁻¹) or a vitamin mixture. Further experi-

**TABLE 1. Comparison of the growth kinetics of members of the haloalkaliphilic iBN-utilizing cocultures**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Soda lake sediment coculture strain ANL-iso2</th>
<th>Soda soil coculture strain ANL-isoa</th>
<th>Soda soil coculture strain ANL-iso4</th>
<th>Soda soil coculture strain ANL-isoa2</th>
</tr>
</thead>
<tbody>
<tr>
<td>iBN</td>
<td>0.035</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Propionitrile</td>
<td>0.035</td>
<td>0</td>
<td>0.14</td>
<td>0</td>
</tr>
<tr>
<td>Butyronitrile</td>
<td>0.030</td>
<td>0</td>
<td>0.28</td>
<td>0</td>
</tr>
<tr>
<td>Valeronitrile</td>
<td>0.030</td>
<td>0</td>
<td>0.23</td>
<td>0</td>
</tr>
<tr>
<td>Capronitrile</td>
<td>0.030</td>
<td>0</td>
<td>0.29</td>
<td>0</td>
</tr>
<tr>
<td>iBA</td>
<td>0.044</td>
<td>0.15</td>
<td>0.19</td>
<td>0.13</td>
</tr>
<tr>
<td>iB</td>
<td>0.045</td>
<td>0.25</td>
<td>0.16</td>
<td>0.33</td>
</tr>
<tr>
<td>Butyrate</td>
<td>0.055</td>
<td>0.22</td>
<td>0.20</td>
<td>0.22</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.050</td>
<td>0.26</td>
<td>0.12</td>
<td>0.66</td>
</tr>
</tbody>
</table>

* Cocultures were grown at pH 10, 0.6 M Na⁺. Values are the averages of the results from two experiments.
  * Growth started after a lag phase of 100 h.
  * Growth started after a lag phase of 200 h.
  * Growth started after a lag phase of 40 h.

**FIG. 2. Growth and product formation with iBN as carbon, energy, and nitrogen source in batch cultures of actinobacterium strain ANL-iso2 (a) and *Bacillus* strain ANL-iso4 (b) at pH 10, 0.6 M Na⁺. Symbols: closed circles, iBN; open circles, iBA; open triangles, NH₃; stars, iB; closed diamonds, biomass. The means of the results from two experiments with deviations of <10% are shown.**
ments proved that a combination of thiamine and biotin was sufficient to replace the yeast extract. The primary hydrolysis of iBN was still possible without vitamins, but the growth of biomass was impaired. The final biomass yield on iBN, iBA, and iB without vitamins was four to five times lower than with the same substrates in the presence of vitamins (Table 2). Artificial reunification of the consortium from two pure cultures restored the complete degradation of iBN and the level of biomass production. According to 16S rRNA gene sequence analysis, both strains belonged to the genus Bacillus, RNA group 1. Bacillus ANL-iso4 forms a separate cluster with an undescribed soil Bacillus sp., BA299 (98% similarity), and with haloalkaliphilic, anaerobic Bacillus arseniciselenatis from Mono Lake, which was its closest culturable relative (95% sequence similarity). Bacillus ANL-iso2 has a 95% sequence similarity to Bacillus arseniciselenatis and bacillus ANL-iso4. Apparently, both strains represent new Bacillus species.

A comparison of the growth characteristics of the two soda soil Bacillus isolates showed some similarities to but also differences from the soda lake sediment isolates. Although the nitrile-degrading member, strain ANL-iso4, was able to grow similarly to ANL-iso2 with the range of aliphatic nitriles (Table 1), its growth kinetics with nitriles were quite different (Fig. 2b). Not only did it grow several times faster, but the biomass growth was parallel to the nitrile hydrolysis, with neither amide nor acid detectable in the culture supernatant, indicating a much more balanced catabolism and anabolism of the nitrile as a growth substrate. Only when the biomass growth was impaired in the absence of vitamins was some accumulation of an acid intermediate detectable in the ANL-iso4 culture (Table 2).

**Influence of pH and salt on growth and iBN degradation activity.** According to the results of pH/salt profiling, all four isolates belonged to moderately salt-tolerant alkaliphiles. The pH ranges for growth at 0.6 M NaCl with iBN were 8.4 to 10.4 (optimum, pH 9.0 to 9.5) for ANL-iso2 and 7.0 to 10.25 (optimum, pH 9.0 to 9.35) for ANL-iso4; with iBA as the substrate, the pH ranges were 8.0 to 10.5 (optimum, pH 9.5) for ANL-iso2 and 7.3 to 10.3 (optimum, pH 9.0) for ANL-iso2. The salt range for growth (M NaCl) at pH 10 was 0.1 to 2 M (optimum, 0.3 to 0.5 M) for all four isolates.

The influence of pH on the levels of nitrile hydratase and amidase activity in the two nitrile-degrading strains was of particular interest. The levels of iBN and iBA hydrolysis activity were tested either with washed cells grown with iBN at pH 10 or with cell extract. Several significant differences between the profiles for whole cells and cell extract and between the pH profiles for activity of ANL-iso2 and ANL-iso4 were observed (Fig. 3). Whole cells clearly tolerated a much broader pH spectrum than the enzymes that were no longer protected by the cell membrane from the external pH condition, although in ANL-iso2, this difference was somewhat less dramatic than in ANL-iso4. Another difference between these two organisms was in the higher alkaline tolerance of the nitrile-degrading enzymes in the actinobacterium.

**Metabolic versatility of haloalkaliphilic isolates.** The growth experiments revealed that the two nitrile-utilizing isolates, an actinobacterium and a Bacillus sp., can metabolize several aliphatic nitriles, most probably through nitrile hydratase activity since both possessed amidase activity and both produced amides from nitriles, although in different proportions. Experiments with washed cells confirmed the presence of a nitrile hydratase/amidase system in both nitrile-degrading strains and also demonstrated that the actual range of substrates being converted is much broader than that utilized for growth (Table 3). An extremely high level of nitrile hydratase activity was observed for C₄ and C₅ aliphatic nitriles, while the level of amidase activity was two to three orders lower. In both nitrile-hydrolyzing strains, nitrile hydratase activity was induced at the same level by either iBN or iBA, while the cells grown on acetate, glucose, or peptone retained only 0 to 10% activity.

## DISCUSSION

Our investigation of iBN degradation by soda lake sediment and soda soil microbial communities demonstrated that iBN degradation is possible in highly haloalkaline conditions and goes through a nitrile hydratase/amidase pathway (Fig. 1). This fact might indirectly indicate that compounds like iBN might be produced as natural substrates in natural environments, for example by the degradation of certain amino acids (7), which would be interesting to test in the future.

As is usually the case with most isolates from soda lake sediments and soda soils, the new bacteria showed typical haloalkaliphilic properties and, as such, represent a new branch of nitrile/amide-hydrolyzing biocatalysts. Whole-cell catalysis is common practice in nitrile bioconversion, since both nitrile hydratase and nitrilase are very unstable enzymes in their free forms. Therefore, further exploration of poten-

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**TABLE 2. Influence of vitamin supplementation and a microbial partner on the efficiency of the growth of nitrile-degrading soil Bacillus strain ANL-iso4 at pH 10**

<table>
<thead>
<tr>
<th>Substrate (mM)</th>
<th>Supplementation or microbial partner</th>
<th>Final biomass (OD₆₀₀)</th>
<th>Final concn iBN (mM)</th>
<th>Maximum concn iBA (mM)</th>
<th>Maximum concn iB (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iBN</td>
<td>Without vitamins</td>
<td>0.10</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>With vitamins</td>
<td>0.59</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>iBA</td>
<td>Without vitamins Bacillus ANL-iso2</td>
<td>0.55</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>With vitamins</td>
<td>0.59</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>iB</td>
<td>Without vitamins</td>
<td>0.10</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>With vitamins</td>
<td>0.63</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* The values are the averages of the results of two experiments with deviations of <10%. NA, not analyzed.
* Vitamins, 10 μg/liter of biotin and thiamine.
* OD₆₀₀, optical density at 600 nm.
tial applications for haloalkaliphilic nitrile-degrading bacteria might be interesting.

Another interesting aspect of this study is the consortial way of utilizing iBN found in both soda lake sediment and soda soil microbial communities, although the type of interaction between the members differs. In the soda lake sediment coculture, only the amide-scavenging member is obligately dependent on the nitrile-hydrolyzing partner. The key to understanding why a coculture was selected instead of a monoculture in this case lies in the growth kinetics parameters of the two organisms (Table 1 and Fig. 2a). The highly disproportionate rates of catabolism of iBN and the further utilization of the products by the actinobacterium allowed the *Marinospirillum*, a scavenger growing several times faster on the degradation products, to efficiently pair with the slowly growing nitrile-hydrolyzing partner (a provider). The fact that the *Marinospirillum* sp. has been selected as a scavenger for iBA might not be accidental, assuming the potential of this particular group to utilize various organic nitrogen compounds originated from anaerobic protein degradation and haloalkaliphily (18).

In contrast, the iBN-utilizing coculture selected from soda soils might be regarded as mutualistic, where both members were obligately dependent on each other. The nitrile-hydrolyzing *Bacillus* strain ANL-iso4 was dependent on growth factors most probably supplied by the second organism, *Bacillus* strain ANL-isoa2, which in turn utilized the products of iBN hydro-

![Graphs showing pH influence on activity](image)

**FIG. 3.** Influence of pH on the activities of the nitrile hydratase/amidase systems in strains ANL-iso2 (a, b) and ANL-iso4 (c, d). (a and c) NH₃ formation from iBN. (b and d) NH₃ formation from iBA. Symbols: closed circles, whole cells; open circles, cell extract. The average values of the results from two independent experiments with deviations of 5 to 10% are shown.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Actinobacterium strain ANL-iso2</th>
<th>Marinospirillum strain ANL-isoa, amidase activity</th>
<th>Bacillus strain ANL-iso4</th>
<th>Bacillus strain ANL-isoa2, amidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nitrile hydratase activity³</td>
<td>Amidase activity³</td>
<td>Nitrile hydratase activity³</td>
<td>Amidase activity³</td>
</tr>
<tr>
<td>Acetonitrile/acetamide</td>
<td>6.2</td>
<td>&lt;0.1</td>
<td>4.6</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Propionitrile/propioamide</td>
<td>16</td>
<td>0.1</td>
<td>1.3</td>
<td>33</td>
</tr>
<tr>
<td>Butyronitrile/butyroamide</td>
<td>50</td>
<td>0.1</td>
<td>0.1</td>
<td>35</td>
</tr>
<tr>
<td>iBN/iBA</td>
<td>24</td>
<td>0.3</td>
<td>0.3</td>
<td>22</td>
</tr>
<tr>
<td>Valeronitrile/valeroamide</td>
<td>58</td>
<td>0.2</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>Capronitrile/caproamide</td>
<td>30</td>
<td>0.1</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Acrylonitrile/acrylamide</td>
<td>28</td>
<td>&lt;0.1</td>
<td>2.2</td>
<td>32</td>
</tr>
<tr>
<td>Benzonitrile/benzoamide</td>
<td>18</td>
<td>&lt;0.1</td>
<td>0</td>
<td>3.1</td>
</tr>
</tbody>
</table>

*Cells were grown with either iBN (strains ANL-iso2 and ANL-iso4) or iBA (strains ANL-isoa and ANL-isoa2) at pH 10. Values are the averages of the results of two or three experiments with deviations within 15%.

³ Amide and acid formation from nitriles at pH 8.

⁴ NH₃ formation from amides at pH 9 to 10.
lysis. Although the latter could be replaced by yeast extract or B-type vitamins, it is still unclear whether other types of interactions were involved in this case as well. Care should be taken, however, in extrapolating from these two examples of bacterial interaction to natural situations with highly heterogeneous conditions, metabolic microdiversity, and much lower concentrations of substrates.

ACKNOWLEDGMENTS

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