Identification of Cyclic Intermediates in \textit{Azorhizobium caulínodans}

Nicotinate Catabolism

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In wild-type \textit{Azorhizobium caulínodans} ORS571, nicotinate served both as anabolic substrate for NAD\(^+\) production and as catabolic substrate for use as the N source. Catabolic enzyme activities were greatest from cultures grown with nicotinate as the N source and least when cultures were grown with ammonium as the N source. Vector insertion mutants unable to catabolize nicotinate (nic::Vi mutants) still required micromolar quantities of this compound for growth. Therefore, \textit{A. caulínodans} wild type is NAD\(^+\) auxotrophic. As the first two intermediates in \textit{A. caulínodans} nicotinate catabolism, two cyclic compounds, 6-hydroxynicotinate and 1,4,5,6-tetrahydro-6-oxonicotinate, were identified. These compounds were purified from the growth medium of strain 61009 (a nic::Vi mutant) by high-performance liquid chromatography; their identities were subsequently confirmed by UV absorbance, nuclear magnetic resonance, and mass spectra. The conversion of 1 mol of nicotinate to 6-hydroxynicotinate consumed 0.5 mol of O\(_2\). From \(^{18}\)O isotopic incorporation experiments, water was the hydroxyl-equivalent source. A nicotinate hydroxylase activity proved to be cell wall-membrane associated; this activity served as direct electron donor (not indirect via NADP\(^+\)) to O\(_2\) via membrane electron transport. These catabolic reactions have not previously been witnessed together in the same organism. \textit{A. caulínodans} nicotinate catabolism seems coupled to N\(_2\) fixation, although the explicit mechanism of this coupling remains to be determined.

Not only does \textit{Azorhizobium caulínodans} ORS571 require nicotinate supplementation for growth in a defined minimal medium, it also uses nicotinate as the sole N source (6, 7). As both a symbiotic and asymbiotic N\(_2\)-fixing strain, \textit{A. caulínodans} is the only characterized member of the Rhizobiaceae clearly able to use N\(_2\) as the N source for its own growth (6). However, because it must be supplemented with nicotinate, which is catabolized under N\(_2\)-fixing conditions, \textit{A. caulínodans} is not a diazotroph. Rather, it conducts synergistic N\(_2\) fixation and nicotinate catabolism (16), as evidenced by the following experiments. When N\(_2\)-fixing \textit{A. caulínodans} cultures under an atmosphere of 97% N\(_2\)-3% O\(_2\) are supplemented with 10-fold-augmented nicotinate levels (16 to 160 \(\mu\)M), a 10-fold increase in cell yield occurs. In contrast, mutants unable to use N\(_2\) as the N source exhibit a negligible increase in growth with increasing nicotinate supplementation (7). In an attempt to elucidate the link between the ability to break down nicotinate and fix N\(_2\), we have investigated \textit{A. caulínodans} nicotinate catabolism in detail.

Nicotinate catabolism has been described in members of the genera \textit{Pseudomonas} (3), \textit{Bacillus} (8), and \textit{Clostridium} (9, 19). Representatives of all three genera first oxidize nicotinate to 6-hydroxynicotinate (6-OH-Nic), but while \textit{Pseudomonas fluorescens} and \textit{Bacillus subtilis} continue to oxidize the pyridine ring structure, \textit{Clostridium barkeri} instead reduces 6-OH-Nic to 1,4,5,6-tetrahydro-6-oxonicotinate (THON) prior to ring breakage.

Whereas most hydroxylases with monooxygenase activity incorporate one atom from O\(_2\) into the product, all studied nicotinate hydroxylases oxidize nicotinate and then rehydrate the product using H\(_2\)O as the hydroxyl-equivalent source. In \textit{C. barkeri} nicotinate fermentation, nicotinate is first oxidized by a cytosolic, NAD\(^+\)-dependent dehydrogenase (12). The \textit{B. subtilis} nicotinate hydroxylase seems to be a soluble, iron-containing flavoprotein that catalyzes O\(_2\) consumption (8, 10, 11). In contrast, \textit{P. fluorescens} and \textit{Pseudomonas putida} (formerly \textit{Pseudomonas ovalis}) nicotinate hydroxylases are membrane-bound complexes, including cytochromes, that catalyze electron transport to O\(_2\) (13–15).

\textit{P. putida} has a nicotinate-driven respiratory chain that terminates at a cytochrome oxidase not reduced by electrons from NADH or succinate (15). In addition, nicotinate hydroxylase and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) both seem to reduce the same cytochrome \(c\) and cytochrome oxidase. This nicotinate-TMPD electron transport chain has been seen in both \textit{P. putida} and \textit{P. fluorescens} (13, 15). In fact, the physiological role of TMPD oxidase activity in \textit{P. putida} is thought to be the hydroxylation of nicotinate. As we show here, \textit{A. caulínodans} seems to combine both \textit{P. putida} aerobic and \textit{C. barkeri} fermentative nicotinate catabolism.

MATERIALS AND METHODS

Bacterial strains and mutants. \textit{A. caulínodans} ORS571 has been previously described (6). A library of vector insertion (Vi) random mutants (carrying IS50-mediated pVPP201 plasmid-genome cointegrates) (5) was screened for members unable to use nicotinate as the sole N source (Nic\(^-\) phenotype). Candidates were collected in three separate experiments. First, nic::Vi 61103, 61007, 61008, and 61009 (Table 1) were isolated by replica plating random Vi mutants onto both rich medium (5) and defined minimal medium (4) containing nicotinate as the sole N source. Colonies that grew on rich medium but not nicotinate-supplemented defined medium were picked; a Nic\(^-\) phenotype was subsequently confirmed by growth tests in similar liquid medium. Approximately five Nic\(^-\) mutants were isolated among 10\(^6\) total Vi mutants. A second protocol was used to isolate mutant strain 61040 (Table 1). Mutagenized cells were first plated on defined medium containing both L-histidine and nicotinate as N sources. Because \textit{A. caulínodans} grows
poorly on L-histidine as the N source, small colonies were picked and candidates were retested for growth in defined liquid medium containing nicotinate as the sole N source. Nic" mutants were isolated at similar frequencies by the two protocols. Only mutant 61009 proved unable to grow on 6-OH-Nic as the sole N source. To isolate additional mutants unable to grow on 6-OH-Nic, the second protocol was repeated using 6-OH-Nic in lieu of nicotinate. Mutant 61020, unable to use 6-OH-Nic as the sole N source, was isolated in this way.

Cell extracts and fractionation procedures. A. caulinodans wild-type cells, cultured in a defined minimal medium containing nicotinate as the sole N source (4), were harvested in late exponential phase by centrifugation at 4,000 × g for 15 min. Cells were suspended in a stabilizing buffer (7 mM sodium potassium phosphate, pH 7.0, 10% glycerol) and then frozen at −70°C until needed. Enzyme activities from rescued cells remained constant over several months of freezing. Thawed cells were lysed by two passes through a French pressure cell at 2,000 lb/in². Unbroken cells were removed by centrifugation at 4,000 × g for 15 min. Cell extracts were centrifuged for 25 min at 300,000 × g in a miniultracentrifuge (model TL100.2; Beckman Instruments, Inc.). On the basis of calculations using the Svedberg equation, only particles with a sedimentation coefficient greater than 30S should have been pelleted. The upper, red, gelatinous pellet layer (envelope fraction) was then suspended in 4 mM NaCl–7 mM sodium potassium diphosphate buffer; the supernatant was considered the cytosolic fraction.

Identification of cyclic nicotinate catabolic intermediates. Cell extracts were diluted to 10 mg ml of total protein−1, and two 300-μl samples were incubated at 37°C. To the experimental sample, nicotinate was added at a final concentration of 0.1%; to the experimental control, phosphate buffer was added. The unmetabolized compound theobromine was used to purify substituents. Under these conditions, THON (0.1 mM) was added to the sample cuvette. Protein was determined by the Bradford method (2). THON degradation by cell extracts and cell fractions was determined by decrease in A250 when THON (0.1 mM) was added to the sample cuvette. Protein was determined by the Bradford method (2).

Reference materials, including nicotinate (Sigma Chemical Co.), 6-OH-Nic (Aldrich Chemical Co.), and [18O]H2O (Amersham Corp.), all were purchased. Nicotinate analogs, 2-chloronicotinate, 6-chloronicotinate, 2-aminonicotinate, 2,4-dihydroxy pyridine, mimosine, and N-methyl nicotinate all were purchased (Sigma). 2,5-Dihydroxy pyridine was the generous gift of E. R. Behrman. Also purchased were the cytochrome oxidase inhibitors sodium cyanide, sodium azide (Sigma), and N-phenylimidazole (Trans World Chemicals).

To purify nicotinate catabolism intermediates, nic::Vi mutant strain 61009 was cultured to late exponential growth phase in defined, minimal medium containing 0.1% 6-OH-Nic and the filtered growth medium was lyophilized. The residue was then suspended in 3 ml of sodium potassium phosphate buffer, passed through a C18 cartridge to remove excess hydrophobic material, and subjected to preparative HPLC. A C18 column (10 by 250 mm; Econosil, Alltech) with a mobile phase of 50 mM ammonium acetate, pH 4.2, was used to purify substituents. Under these conditions, THON exhibited a 10-min-longer retention time relative to all other absorbance peaks. Fractions containing THON were pooled and lyophilized, and the residue was suspended in 1 ml of water. This solution was acidified to pH 1.0 with reagent grade sulfuric acid and then extracted for 24 h with 50 ml of diethyl ether. Ether phases were evaporated at room temperature under argon, and the resulting white powder residue was dissolved in deuterated dimethyl sulfoxide for measurement of proton magnetic resonance spectra. Nuclear magnetic resonance was performed in a 300-MHz General Electric GN300 machine. A Finnegan mass spectrometer with either methane or isobutane as the ionizing gas was used for molecular weight determinations. UV absorbance scans were performed on a Hitachi 100-80 spectrophotometer. Atomic composition was assayed with a Perkin-Elmer 240B element analyzer.

Enzyme assays and chemicals. Strains were tested for TMPD oxidase activity by rubbing a picked colony on filter paper saturated with 5% TMPD in water. Blue color in 1 to 2 min indicates a positive response; colony smears remaining white for 20 to 30 min were considered negative. Acetylene reduction assays and nodulation studies on the host plant Sesbania rostrata were performed as previously described (4). All O2 consumption experiments were performed in a modified Warburg apparatus at 30°C. Vials contained whole cells, 7 mM sodium potassium phosphate buffer, and substrate adjusted to a 2.4-ml final volume. 6-OH-Nic has an absorbance peak at 295 nm (e = 3.6 × 104) at which nicotinate absorbs negligibly (12, 13). Therefore, nicotinate hydroxylase activity was assayed by following increase in A250 in a Hitachi 100-80 recording spectrophotometer at room temperature (about 23°C). Both the sample and reference cuvettes contained cell fractions diluted to 1 ml in 7 mM sodium potassium phosphate buffer, pH 7.0; the sample cuvette contained 1 mM nicotinate. Cytochrome oxidase inhibitors and electron acceptors were added to both cuvettes.

Change in A440 was used to detect reduction and oxidation of both NADPH and NADH. Methylene blue and ferricyanide reductions were followed at A600 and A450, respectively. THON degradation by cell extracts and cell fractions was determined by decrease in A250 (a peak for THON at pH 7; e = 1.4 × 105) when THON (0.1 mM) was added to the sample cuvette. Protein was determined by the Bradford method (2).

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RESULTS

*A. caulinodans* mutant and wild-type growth properties. Six *A. caulinodans* nic::*Vi* mutants, strains 61007, 61008, 61009, 61020, 61040, and 61103, were isolated and phenotypically characterized (Table 1). Two mutants, strains 61009 and 61020, were unable to use 6-OH-Nic as the N source but were unaffected in nicotinate hydrolyase activity, N$_2$ fixation, and *S. rostrata* nodulation. Among those mutants able to utilize 6-OH-Nic as a N source, strains 61040 and 61103 were also unable to oxidize TMPD and were impaired in both N$_2$ fixation and nodulation abilities. The nic::*Vi* mutants 61007 and 61008 had little or no nicotinate hydroxylase activity and were generally unimpaired in both N$_2$ fixation and nodulation.

*A. caulinodans* wild type and all mutants required at least 1 μM nicotinate for good growth on any N source, including 6-OH-Nic. When defined medium contained L-glutamine as the sole N source, wild-type cultures doubled their cell counts every 3 h; cultures doubled every 11 h with nicotinate as the sole N source. This finding is consistent with the low rates of enzyme activity seen in cell extracts (see below).

Because *A. caulinodans* wild type grew poorly on either nicotinate or 6-OH-Nic as the C source, experiments attempting to show growth on potential (N-free) ring breakage products proved ambiguous. As C substrates for *A. caulinodans* wild-type growth, fumarate, propionate, and glucose are well as compound(s) eluting at 10 min but did not yield any UV-absorbing compounds eluting at 2.8 min.

When the mutant strain 61009 was cultured in defined medium supplemented with excess 6-OH-Nic, large amounts of the compound(s) eluted at 10 min (see above) were obtained after preparative HPLC (Materials and Methods). The proton NMR spectrum of the principal, purified compound closely resembled that reported for THON produced during *C. barkeri* nicotinate fermentation (9, 19). The UV absorbance spectrum for this compound had an absorbance peak at 275 nm in acidic solution that shifted to 268 nm in a basic solution and was identical to that of THON. From mass spectrometry, a molecular weight of 141 was obtained for this compound; elemental analysis was consistent with a molecular formula of C$_9$H$_7$NO$_3$ (Table 2).

As isolated from mutant 61009, THON would not serve as the sole N source for *A. caulinodans*. However, THON degradation was detected in *A. caulinodans* cell extracts and occurred at rates on the same order of magnitude as rates of nicotinate hydroxylation (discussed below). Neither strain 61009 nor the wild type cultured with ammonium as the N source showed significant THON breakdown activity (see Table 4).

**O$_2$ consumption during *A. caulinodans* nicotinate catabolism.** Using a modified Warburg apparatus (Materials and Methods), O$_2$ consumption was measured in *A. caulinodans* wild-type cells previously grown in defined medium with nicotinate as the sole N source. While complete combustion of 1 mol of nicotinate would require 5.5 mol of O$_2$, only 3.5 mol of O$_2$ was consumed in this experiment (Fig. 1). In a parallel control experiment, only 2.5 mol of O$_2$ was consumed per mol of succinate; in both cases, some substrate may have been used in anabolic reactions.

### Table 2. Comparison between results of two studies

<table>
<thead>
<tr>
<th>THON</th>
<th>Proton NMR peaks (ppm)*</th>
<th>HCl</th>
<th>NaOH</th>
<th>Composition</th>
<th>Mol wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_{max}$ (nm)</td>
<td>$\epsilon \times 10^4$</td>
<td>$\lambda_{max}$ (nm)</td>
<td>$\epsilon \times 10^4$</td>
<td></td>
</tr>
<tr>
<td>Previously reported$^b$</td>
<td>2.6, $^c$ 7.3$^d$</td>
<td>277</td>
<td>1.40</td>
<td>268</td>
<td>1.40</td>
</tr>
<tr>
<td>This study</td>
<td>2.5, $^c$ 7.1$^d$</td>
<td>275</td>
<td>1.45</td>
<td>268</td>
<td>1.4</td>
</tr>
</tbody>
</table>

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$^*$ Tsai et al. (18) used DCCI as the solvent, and we used deuterated dimethyl sulfoxide, which may account for the slight difference in peak positions. NMR, Nuclear magnetic resonance.

$^b$ As reported by Tsai et al. (18).

$^c$ A multiplet for both; a signal from the protons on C-4 and C-5.

$^d$ A doublet for both; a signal from the proton on C-2.
grown in defined medium with nicotinate as the sole N source, added to each Warburg vial. Plots using nicotinate (○) or succinate (■) as the substrate had the O\textsubscript{2} consumption of a control with no substrate subtracted from them. Substrate (2 \textmu mol) was added in all cases; each plot is an average of two parallel experiments.

When strain 61009 was similarly tested, 0.5 mol of O\textsubscript{2} per mol of nicotinate was consumed; no O\textsubscript{2} was consumed at the expense of 6-OH-Nic (Fig. 2). Because more O\textsubscript{2} was consumed per mol of nicotinate than per mol of succinate and because the conversion of nicotinate to THON required only a single two-electron oxidation, some ring breakage products must be further oxidized by A. caulinodans (see Discussion).

**Nicotinate hydroxylase incorporates \[{}^{18}\text{O}\]H\textsubscript{2}O into 6-OH-Nic.** After overnight incubation with 1% nicotinate in a 20% \[{}^{18}\text{O}\]H\textsubscript{2}O solution, mutant 61009 cultures yielded 6-OH-Nic with a 20% increase in signal at m/z = 142, as compared with the 6-OH-Nic isolated from a light-water-nicotinate solution (Fig. 3). Using isobutane as the ionizing gas shifts all molecular weight readings to M+1; thus, 6-OH-Nic gave a maximum at m/z = 140 instead of 139. No solvent exchange of 6-OH-Nic hydroxyl groups was detected. When purified, 20% \[{}^{18}\text{O}\]-6-OH-Nic was incubated in light-water solution for 2 days; the proportion of heavy 6-OH-Nic remained 20%.

**Localization of nicotinate-induced A. caulinodans nicotinate hydroxylase activity to the cell envelope.** As measured by the production of 6-OH-Nic (Materials and Methods), nicotinate hydroxylase activity was found to be concentrated in the cell envelope fraction of cell extracts. A small amount of nicotinate hydroxylase activity was also evident in the wild-type cell extract cytosolic fraction (Table 3) which may have been contaminated with bits of cell envelope. The NADH oxidation rate by the envelope fraction was 10-fold that of nicotinate oxidation (data not shown). With cells grown on other N sources, nicotinate hydroxylation rates decreased dramatically (Table 4). Moreover, rates of THON breakdown, measured by the disappearance of THON (Materials and Methods), also decreased. Unlike nicotinate hydroxy-

**DISCUSSION**

When catabolizing nicotinate, A. caulinodans and all other similarly investigated bacteria produce, as an initial intermediate, 6-OH-Nic using H\textsubscript{2}O as the hydroxyl-equivalent source (3, 8, 9; this study). Whereas other aerobic organisms next oxidize 6-OH-Nic to yield 2,6-dihydroxynicotinate or 2,5-dihydroxyxypyridine (3, 8), A. caulinodans instead reduces 6-OH-Nic to THON (this study). Because THON breakdown and its regulation coordinated with nicotinate hydroxylase activities in both the nic::V\textsubscript{i} mutant and wild-type analyses above, THON is a true A. caulinodans nicotinate catabolic intermediate. An analogous reduction of 6-OH-Nic to THON has been reported for C. barkeri during anaerobic fermentations (9, 19).

Surprisingly, A. caulinodans seems to share its nicotinate catabolic properties with the obligate anaerobic Clostridium...
Table 3. 6-OH-Nic production of A. caulinodans cells grown on nicotine as the sole N source

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg ml⁻¹)</th>
<th>Total activity (mmol min⁻¹)</th>
<th>Sp act (mmol min⁻¹ mg of protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysate</td>
<td>42</td>
<td>44</td>
<td>1.4</td>
</tr>
<tr>
<td>Cell envelope</td>
<td>11</td>
<td>28</td>
<td>4.4</td>
</tr>
<tr>
<td>Cytosol</td>
<td>30</td>
<td>10</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table 4. Nicotinate hydroxylase and THON breakdown activities in cell extracts prepared from cultures grown with several N sources

<table>
<thead>
<tr>
<th>N source</th>
<th>Nicotinate hydroxylase (mmol min⁻¹ mg of protein⁻¹)</th>
<th>THON breakdown (mmol min⁻¹ mg of protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₂</td>
<td>0.36</td>
<td>0.9</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>0.14</td>
<td>0.2</td>
</tr>
<tr>
<td>Nicotinate</td>
<td>1.4</td>
<td>0.45</td>
</tr>
</tbody>
</table>

caulinodans continues to mimic Clostridium spp. nicotinate catabolism, ring breakage would produce ammonium and 2-methylglutarate, which would be converted stoichiometrically into equal amounts of pyruvate and propionate (19). By analogy to odd-chain fatty acid oxidation, propionate might be carboxylated to eventually produce succinate (Fig. 4).

In Clostridium spp., nicotinate hydroxylation is conducted by a soluble, NAD⁺-dependent dehydrogenase (12). However, A. caulinodans nicotinate hydroxylase activity was membrane associated and seemed to require O₂, not NAD⁺, as the terminal electron acceptor. Nicotinate hydroxylase and NADH dehydrogenase activities were inhibited by azide to strikingly different degrees (Table 5). In addition, neither mutant 61103 nor 61040 could oxidize TMPD (Table 1). Analogously, hydroxylation of nicotinate in P. putida and P. fluorescens is membrane bound and coincides with TMPD oxidation (13, 14, 15). Both substrates reduce an electron transport chain other than that involving NADH dehydrogenase, as evidenced by cytochrome spectra and inhibitor studies with azide in P. putida (15). Thus, A. caulinodans may have a nicotinate electron transport system similar to these pseudomonads.

A. caulinodans nicotinate catabolism seems to have borrowed from both aerobic and anaerobic pathways. However, this revelation sheds little light on how degradation of this essential vitamin enhances N₂ fixation. From this study, neither nicotinate nor 6-OH-Nic catabolism seems essential for N₂ fixation. Under N₂-fixing conditions, increased nicotine levels might produce increased growth yields simply because competitive nicotinate catabolism depletes cells of the vitamin. Regardless, mutants 61103 and 61040 are impaired in both free-living and symbiotic N₂ fixation (Table 1). Intriguingly, Bradyrhizobium japonicum and Rhizobium phaseoli mutants isolated by their inability to oxidize TMPD are either unable to nodulate their host plants or produce ineffective (Fix⁻) nodules (17, 18). Since the A. caulinodans Nic- mutants 61103 and 61040 were also unable to oxidize...
TMPD, the electron transport chain necessary for nicotinate hydroxylation and TMPD oxidation must contribute in some way to an intracellular environment conducive to high levels of N₂ fixation.

ACKNOWLEDGMENTS

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LITERATURE CITED