Identification of Cyclic Intermediates in\textit{Azorhizobium caulinodans}\n
Nicotinate Catabolism

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In wild-type \textit{Azorhizobium caulinodans} 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. caulinodans} wild type is NAD$^+$ auxotropic. As the first two intermediates in \textit{A. caulinodans} 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 a 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. caulinodans} nicotinate catabolism seems coupled to N$_2$ fixation, although the explicit mechanism of this coupling remains to be determined.

Not only does \textit{Azorhizobium caulinodans} 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. caulinodans} 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. caulinodans} 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. caulinodans} 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. caulinodans} nicotinate catabolism in detail.

Nicotinate catabolism has been described in members of the genera Pseudomonas (3), Bacillus (8), and Clostridium (9, 19). Representatives of all three genera first oxidize nicotinate to 6-hydroxynicotinate (6-OH-Nic), but while \textit{Pseudomonas fluorescens} and 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 monoxygenase 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 \texti{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. caulinodans} seems to combine both \textit{P. putida} aerobic and \textit{C. barkeri} fermentative nicotinate catabolism.

MATERIALS AND METHODS

Bacterial strains and mutants. \textit{A. caulinodans} ORS571 has been previously described (6). A library of vector insertion (Vi) random mutants (carrying IS50-mediated pVP2021 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$^8$ 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. caulinodans} grows...
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 C_{18} cartridge to remove excess hydrophobic material, and subjected to preparative HPLC. A C_{18} 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. Either phases were evaporated at room temperature under argon, and the resulting white power 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.

**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 O$_2$ 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 ($\epsilon = 3.6 \times 10^3$) at which nicotinate absorbs negligibly (12, 13). Therefore, nicotinate hydroxylase activity was assayed by following increase in A$_{295}$ 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 A$_{440}$ was used to detect reduction and oxidation of both NADPH and NADH. Methylen blue and ferricyanide reductions were followed at A$_{600}$ and A$_{450}$, respectively. THON degradation by cell extracts and cell fractions was determined by decrease in A$_{270}$ (a peak for THON at pH 7; $\epsilon = 1.4 \times 10^5$) 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 $[^{14}C]$O$_2$(OAmersham Corp.), all were purchased. Nicotinate analogs, 2-chloronicotinate, 6-chloronicotinate, 2-amino nicotinate, 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).
time. The absorbance peak eluting at 3.5 min was identified nicotinate was added to extracts, three UV-absorbing peaks and THON. To identify compounds produced during nico-
tinate catabolism, A. caulinodans used any of these compounds as the sole N source, wild-type cultures doubled their cell growth in defined medium with nicotinate as the sole N source, whereas the marker compound theobromine eluted at 3.2 min. Aside from theobromine, no peaks appeared in the

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 neither could As isolated from mutant 61009, THON would not serve as a N source but did not inhibit 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) eluting 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 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 C₆H₆NO₃ (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).

O₂ consumption during A. caulinodans nicotinate catabolism. Using a modified Warburg apparatus (Materials and Methods), O₂ 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 O₂, only 3.5 mol of O₂ was consumed in this experiment (Fig. 1). In a parallel control experiment, only 2.5 mol of O₂ was consumed per mol of succinate; in both cases, some substrate may have been used in anabolic reactions.

<table>
<thead>
<tr>
<th>THON</th>
<th>Proton NMR peaks (ppm)*</th>
<th>HCl</th>
<th></th>
<th>NaOH</th>
<th></th>
<th>Composition</th>
<th>Mol wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λ_max (nm)</td>
<td>ε (10^4)</td>
<td>λ_max (nm)</td>
<td>ε (10^4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previously reported&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.6, 3.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>277</td>
<td>1.40</td>
<td>268</td>
<td>1.40</td>
<td>C₆H₆NO₃</td>
<td>141</td>
</tr>
<tr>
<td>This study</td>
<td>2.5, 7.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>275</td>
<td>1.45</td>
<td>268</td>
<td>1.4</td>
<td>C₆H₆NO₃</td>
<td>141</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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.

<sup>b</sup> As reported by Tsai et al. (18).

<sup>c</sup> A multiplet for both; a signal from the protons on C-4 and C-5.

<sup>d</sup> A doublet for both; a signal from the proton on C-2.
When strain 61009 was similarly tested, 0.5 mol of O$_2$ per mol of nicotinate was consumed; no O$_2$ was consumed at the expense of 6-OH-Nic (Fig. 2). Because more O$_2$ 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).

**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 cytokinesis 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 hydroxy­

**Discussion**

When catabolizing nicotinate, *A. caulinodans* and all other similarly investigated bacteria produce, as an initial intermediate, 6-OH-Nic using H$_2$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-dihydroxyxypyrindine (3, 8), *A. caulinodans* instead reduces 6-OH-Nic to THON (this study). Because THON break­

Surprisingly, *A. caulinodans* seems to share its nicotinate catabolic properties with the obligate anaerobic *Clostridium*
Further oxidized to account for the amount of O₂ consumed this pyridine ring, only 0.5 mol of the 3.5 mol of O₂ consumed per mol of nicotinate can be attributed to reac-

per mol of nicotinate. Since mutant 61009 did not catabolize the vitamin. Regardless, mutants 61103 and 61040 are im-

paired 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

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 hydroxylation activity was membrane associated and seemed to require O₂, not NAD⁺, as the terminal electron acceptor. Nicotinate hydroxylation 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 nicotinate levels might produce increased growth yields simply because competitive nicotinate catabolism depletes cells of the vitamin. Regardless, mutants 61103 and 61040 are im-

paired in both free-living and symbiotic N₂ fixation (Table 1).

TABLE 3. 6-OH-Nic production of A. caulinodans cells grown on nicotinate 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>

FIG. 3. Cells of the nic::Vi mutant 61009 were grown overnight with glutamate as the N source, induced for nicotinate catabolic enzymes by the addition of excess nicotinate for 4 h, and harvested by centrifugation. Cells (2 g) were then incubated for 24 h in a 5-ml solution of 1% nicotinate, with or without [¹⁸O]H₂O. Cells were removed by centrifugation and filtration. The 6-OH-Nic in the filtrate was isolated by preparative HPLC and mass spectra were taken. (A) Mass spectrum of 6-OH-Nic after incubation with [¹⁸O]H₂O. (B) Mass spectrum of 6-OH-Nic after incubation with [¹⁸O]H₂O.

sp. and not with the more closely related aerobes. But because infected plant tissue constitutes a microaerobic environment that is eventually O₂ buffered at submicromolar concentrations by leghemoglobin (1), A. caulinodans redox-balanced nicotinate catabolism might allow complete nicotinate degradation to tricarboxylic acid cycle intermediates which can be efficiently stored as poly-3-hydroxybutyrate (20).

Although nicotinate metabolites after ring breakage were not characterized, some ring breakage products must be further oxidized to account for the amount of O₂ consumed per mol of nicotinate. Since mutant 61009 did not catabolize this pyridine ring, only 0.5 mol of the 3.5 mol of O₂ consumed per mol of nicotinate can be attributed to reactions occurring before ring breakage (see Results). If A. 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).

TABLE 5. Effects of inhibitors on both nicotinate hydroxylase and NADH dehydrogenase activities measured in crude cell envelope fractions

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc (mM)</th>
<th>Nicotinate hydroxylase inhibition (%)</th>
<th>NADH dehydrogenase inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN⁻</td>
<td>2.5</td>
<td>79</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>86</td>
<td>95</td>
</tr>
<tr>
<td>N-Phenylimidazole</td>
<td>2.5</td>
<td>30</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>52</td>
<td>64</td>
</tr>
<tr>
<td>N₃⁻</td>
<td>2.5</td>
<td>6</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>16</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>45</td>
<td>73</td>
</tr>
</tbody>
</table>
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