Elucidation of the Complete *Azorhizobium* Nicotinate Catabolism Pathway

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A complete pathway for *Azorhizobium caulinodans* nicotinate catabolism has been determined from mutant phenotype analyses, isolation of metabolic intermediates, and structural studies. Nicotinate serves as a respiratory electron donor to O_2 via a membrane-bound hydroxylase and a specific *c*-type cytochrome oxidase. The resulting oxidized product, 6-hydroxynicotinate, is next reduced to 1,4,5,6-tetrahydro-6-oxonicotinate. Hydrolytic ring breakage follows, with release of pyridine N as ammonium. Decarboxylation then releases the nicotinate C-7 carboxyl group as CO_2, and the remaining C skeleton is then oxidized to yield glutarate. Transthioesterification with succinyl coenzyme A (succinyl-CoA) yields glutaryl-CoA, which is then oxidatively decarboxylated to yield crotonyl-CoA. As with general acyl β oxidation, L-β-hydroxybutyryl-CoA, acetoacetyl-CoA, and finally two molecules of acetyl-CoA are produced. In sum, nicotinate is catabolized to yield two CO_2 molecules, two acetyl-CoA molecules, and ammonium. Nicotinate catabolism stimulates *Azorhizobium* N_2 fixation rates in culture. Nicotinate catabolism mutants still able to liberate pyridine N as ammonium retain this capability, whereas mutants so blocked do not. From, mutant analyses and additional physiological tests, N_2 fixation stimulation is indirect. In N-limited culture, nicotinate catabolism augments anabolic N pools and, as a consequence, yields N_2-fixing cells with higher dinitrogenase content.

Azorhizobium caulinodans invades both stems and roots of the host legume *Sesbania rostrata* and yields symbiotic nodules that fix N_2 at very high rates. All wild *Azorhizobium* isolates are phenotypically indistinguishable and are auxotrophic for pyridine nucleotide cofactor (NAD^+) biosynthesis; culture in defined medium requires nicotinate supplementation (10). When so cultured, *A. caulinodans* also uses nicotinate as the sole N source for growth (22). Whereas all other studied rhizobia exclusively fix N_2 in symbiosis, *A. caulinodans* avidly fixes N_2 in culture (10). Paradoxically, when fixing N_2 in culture under optimal conditions, *A. caulinodans* exhaustively catabolizes nicotinate, and its growth becomes NAD^+ limited. Relative to a submicromolar nicotinate vitamin requirement, high-level (0.3 mM) nicotinate supplementation strongly stimulates N_2 fixation in culture (4, 11). We have sought to understand how nicotinate stimulates N_2 fixation.

Although nicotinate is a good N source, it is a poor C source for *Azorhizobium* growth (18). However, stoichiometric measurements of nicotinate-stimulated O_2 consumption indicate that *A. caulinodans* completely utilizes the nicotinate C skeleton (18). Cyclic intermediates in this pathway were previously isolated and characterized, and a mechanism of pyridine ring N release was proposed (18) (Fig. 1). Nicotinate is first oxidized to yield 6-hydroxynicotinate by a membrane-bound hydroxylase, which uses O_2 as an electron acceptor. The nicotinate-specific respiratory chain to O_2 terminates at a *c*-type cytochrome oxidase (17). The reaction product, 6-hydroxynicotinate, is next reduced to form 1,4,5,6-tetrahydro-6-oxonicotinate (THON) by a soluble dehydrogenase activity (18). As with the obligate anaerobe *Clostridium barkeri* (14, 28), the THON pyridine ring is next cleaved, and ammonium is released, by hydrolysis with two water equivalents. Further catabolic steps in the two bacteria then diverge. *C. barkeri* ferments nicotinate, undertaking a complex C-skeleton rearrangement without decarboxylation, to yield pyruvate and propionate (28). *A. caulinodans*, as described here, catabolizes THON by utilizing two oxidative decarboxylations to yield acetyl coenzyme A (acetyl-CoA) (Fig. 1).

Azorhizobium insertion mutants with altered nicotinate hydroxylase activity were previously isolated by screening for candidates unable to use nicotinate as an N source (Nic[N] phenotype). These mutants were then subclassified according to their ability to use 6-hydroxynicotinate as an N source (6HN[N] phenotype) (4, 18). When nicotinate levels in N_2-fixing batch cultures were increased from 16 μM to 0.3 mM, all *Azorhizobium* $Nic[N]^-$ strains, in contrast to the wild type, showed no stimulation of dinitrogenase activity. However, for $Nic[N]^-$ the $6HN[N]^+$ mutants, dinitrogenase activity was fully stimulated when 0.3 mM 6-hydroxynicotinate was added to these cultures. In contrast, N_2 fixation by $6HN[N]^-$ mutants did not respond to 6-hydroxynicotinate supplementation (4). Therefore, stimulation of N_2 fixation correlates with a nicotinate catabolism event(s) subsequent to 6-hydroxynicotinate production and not with nicotinate hydroxylase-driven respiration.

As reported here, *Azorhizobium* mutants affected in distal steps of the nicotinate catabolic pathway nevertheless conduct abortive nicotinate catabolism, release pyridine N as ammonium, and remain able to grow on nicotinate as an N source. These mutants fix N_2 with wild-type physiological properties. From these and other experiments, by inference, nicotinate catabolism indirectly stimulates N_2 fixation by augmenting steady-state anabolic N pools in N-limited culture.

MATERIALS AND METHODS

Bacterial strains, mutant isolations, and growth media. *A. caulinodans* wild-type strain 57100 (9) was mutagenized by the vector insertion (Vi) technique as previously described (8); a random spectrum of mutants was obtained (7). A

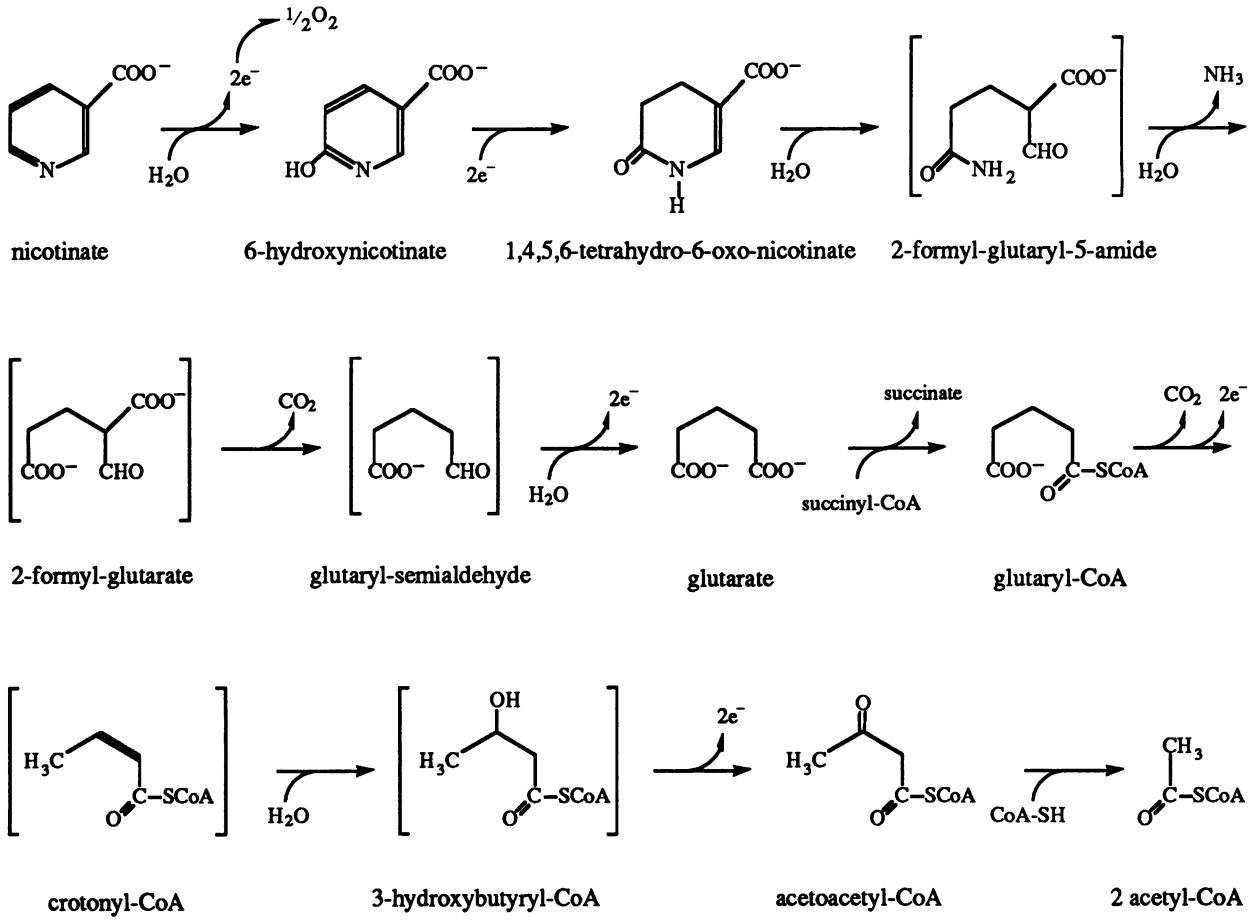

FIG. 1. The *Azorhizobium* nicotinate catabolic pathway. Intermediates in brackets are inferred from the data presented; not all intermediates may be released as free, diffusible compounds (18).

library of 600,000 random Vi mutants was screened for inability to use either DL-β-hydroxybutyrate or 6-hydroxynicotinate as a C source (βHB$^-$ or 6HN[C]$^-$ phenotype). Candidates that remained able to grow with succinate as a C source on plates were purified and retested for C-source utilization in defined liquid medium. Defined growth medium consisted of minimal salts (basal medium) supplemented with 20 mM C source and 10 mM N source (4). C sources tested were succinate, glutarate, 6-hydroxynicotinate, DL-β-hydroxybutyrate, acetoacetate, and acetate; N sources tested were glutamate, ammonium, nicotinate, and 6-hydroxynicotinate.

Physiological radiolabeling studies. Bacterial strains were cultured in basal medium with succinate as a C source and glutamate as an N source; cells were harvested by centrifugation and washed once in P7 buffer (7 mM potassium phosphate buffer [pH 7.0]) and diluted in 80 mM phosphate buffer [pH 7.0] to an A_{600} of 0.4. Samples (5 ml) were added to serum vials containing 1-ml 1 M NaOH wells, and physiological labeling was initiated with 0.1 mM [7-^{14}C]nicotinate (0.7 μCi μmol^{-1}). Serum vials were incubated for 3 h at 30°C on a rotary shaker, brought to pH 1.0 with 0.2 ml concentrated H_2SO_4, and further shaken for 6 to 8 h. Both cell suspension-assimilated ^{14}C and unincorporated, trapped ^{14}C were then counted by Cerenkov scintillation, and fractional assimilation was determined.

SGCT and GCADH assays. Cultures in mid-exponential growth phase were harvested by centrifugation, washed once in P7 buffer, concentrated 100-fold, and lysed by ultrasonication. Unbroken cells were removed by centrifugation, and total protein in cell extracts was determined by the method of Bradford as described elsewhere (1). Reactions were carried out in 1-ml cuvettes containing 50 mM Tris-Cl (pH 8.0), 2 mM ferricyanide, 10 mM sodium cyanate, and 50 μl of cell extract. Glutaryl-CoA dehydrogenase (GCADH) activity was recorded as 2 mM glutaryl-CoA (Sigma Chemical Co.)-dependent decrease in A_{420}. Succinate:glutarate-CoA transferase (SGCT) activity was assayed similarly, with 0.5 mM succinyl-CoA (Sigma) added. Activity was recorded as 10 mM glutarate-dependent decrease in A_{420}.

Dinitrogenase assays. Bacteria were cultured to early exponential growth phase in defined liquid basal medium supplemented with the indicated C and N sources (see text) under 21% O_2, washed, and then resuspended in NIF (4) medium under 3% O_2; N_2 fixation is optimum in these conditions (4). After cultures were fully induced (8 to 10 h), stoppered vials were injected with acetylene to 0.01 atm by using a gas-tight syringe. Ethylene production was measured at time zero and hourly for 3 h by gas chromatography (4), and data were extrapolated to infer initial velocities. Light scattering (A_{600}) by induced cultures was also measured, and

TABLE 1. *A. caulinodans* Nic$^-$ Vi mutants and their phenotypes

Strain[a]	Phenotype						
	6HN[N]	6HN[C]	deCO$_2$[b]	Glr	βHB	Aac	Ace
57100 (wild type)	+	+	+	+	+	+	+
Mutants							
61606 (5)	+	−	−	+	+	+	+
61604	+	−	−	+	+	+	+
61607	+	−	−	+	+	+	+
61612	+	−	−	+	+	+	+
61618	+	−	−	+	+	+	+
61505 (3)	+	−	+	+	+	+	+
61504	+	−	+	+	+	+	+
61509	+	−	+	+	+	+	+
61510 (4)	+	−	+	−	+	+	+
61515	+	−	+	−	+	+	+
63077 (5)	+	−	+	−	−	−	+
63083 (16)	+	−	+	−	−	−	−

[a] Nicotinate mutant strains are divided by phenotypic subclass, with the archetype strain listed first. All mutants carry single VP2021 insertions. Numbers of independent mutants are listed in parentheses.
[b] Wild-type [7-^{14}C]nicotinate decarboxylase activity.

absorbances were converted to cells per milliliter by interpolation with a standard curve of light scattering (A_{600}) versus viable cell count.

RESULTS

Isolation of *Azorhizobium* βHB$^-$ or 6HC[C]$^-$ mutants. An *A. caulinodans* 57100 library carrying random, single VP2021 insertions (Materials and Methods) was screened for candidates unable to use DL-β-hydroxybutyrate as a C source for growth; 21 independent mutants were identified (Table 1). All 21 mutants unable to utilize racemic DL-β-hydroxybutyrate also proved unable to utilize 6-hydroxynicotinate as a C source. These mutants implicate distal steps in nicotinate catabolism, as discussed below. The same library, minus the DL-β-hydroxybutyrate mutant candidates, was further screened for derivatives unable to utilize 6-hydroxynicotinate as a C source (6HN[C]$^-$). Those candidates also unable to use 6-hydroxynicotinate as an N source (6HN[N]$^-$) were identified and disregarded. Twelve independent candidates resulted; all proved able to use both nicotinate and 6-hydroxynicotinate as the sole N source (Nic[N]$^+$ 6HN[N]$^+$) but unable to use 6-hydroxynicotinate as a C source (6HN[C]$^-$). All mutants were then categorized by ability to grow on four C sources: acetate, acetoacetate, DL-β-hydroxybutyrate, and glutarate (Table 1). All 6HN[C]$^-$ mutants unable to use acetate as a C source (Ace$^-$) could use neither acetoacetate (Aac$^-$) nor glutarate (Glr$^-$) as a C source. Similarly, all Aac$^-$ Ace$^+$ mutants also proved to be Glr$^-$. Four independent Glr$^-$ Aac$^+$ Ace$^+$ mutants were identified (Table 1). Compared with the wild type, the remaining eight mutants all used the four C sources normally. These mutants were eventually subclassified by analysis of their decarboxylase activities, as discussed below. All 6HN[C]$^-$ Aac$^+$ mutants remained able to use both D-β-hydroxybutyrate and L-β-hydroxybutyrate as C sources.

During its catabolism, nicotinate suffers decarboxylative loss of the C-7 carboxyl group. Eight 6HN[N]$^+$ 6HN[C]$^-$ Glr$^+$ mutants were tested for ability to release $^{14}CO_2$ from [7-^{14}C]nicotinate (Materials and Methods). As it is unable to break the nicotinate ring (4), 6HN[N]$^-$ strain 61309 was included as a deficient experimental control. Strain 61510 (6HN[N]$^+$ 6HN[C]$^-$ Glr$^-$) and wild-type strain 57100 were also included as proficient experimental controls. For all samples, percentage total $^{14}CO_2$ released and trapped as sodium carbonate was determined by scintillation spectrometry (Table 2; Materials and Methods). Conditioned growth media of all strains except strain 61309 showed no UV absorbance at 257 nm above background. Thus, nicotinate was exhausted at the end of the experiment. Because strain 61309 cultures accumulate THON in growth media and lack THON hydrolase activity (18), the small amount of $^{14}CO_2$ produced by strain 61309 may reflect an acid-labile THON carboxyl group. Five of the eight 6HN[N]$^+$ 6HN[C]$^-$ Glr$^+$ mutants released less than 60% of added [7-^{14}C]nicotinate as $^{14}CO_2$; the three other mutants released more than 95%. Unfortunately, no 6HN[N]$^+$ 6HN[C]$^-$ mutant tested retained more than 60% of the [7-^{14}C]nicotinate label. As discussed below, hydrolysis of THON with concomitant ammonium release yields 2-formylglutarate (2FG) (28). Because the experimental media were acidified to volatilize CO_2, any produced 2FG might undergo spontaneous decarboxylation of its C-1 carboxyl group (corresponding to nicotinate C-7) at appreciable rates. Correspondingly, $^{14}CO_2$ release from [7-^{14}C]nicotinate would inevitably be substantial relative to that observed with strain 61309, in which ring breakage does not occur. Because they released less than 60% of total [7-^{14}C]nicotinate as $^{14}CO_2$, these six mutants may be unable to enzymatically decarboxylate nicotinate.

TABLE 2. *Azorhizobium* physiological radiolabeling with [7-^{14}C]nicotinate

Strain	Phenotype	% $^{14}CO_2$ released[a]
57100	Wild type	97
61309	6HN[N]$^-$	16
61604	6HN[N]$^+$ 6HN[C]$^-$	56
61606	6HN[N]$^+$ 6HN[C]$^-$	44
61607	6HN[N]$^+$ 6HN[C]$^-$	58
61612	6HN[N]$^+$ 6HN[C]$^-$	56
61618	6HN[N]$^+$ 6HN[C]$^-$	54
61505	6HN[N]$^+$ 6HN[C]$^-$	96
61504	6HN[N]$^+$ 6HN[C]$^-$	97
61509	6HN[N]$^+$ 6HN[C]$^-$	96
61510	6HN[N]$^+$ Glr$^-$	98

[a] Percent total radiolabel recovered as CO_2 (Materials and Methods).

2FG might suffer either of two catabolic fates: it might be decarboxylated to yield glutaryl semialdehyde and then oxidized to yield glutarate, or it might be oxidatively decarboxylated to directly yield glutarate. While we attempted to distinguish between these two possibilities, we could neither obtain nor synthesize glutaryl semialdehyde. In either case, if glutarate were produced by nicotinate catabolism, then 6HN[N]$^+$ Glr$^-$ mutant cultures might accumulate glutarate in conditioned media.

6-Hydroxynicotinate catabolism yields glutarate. Strain 61510 (Glr$^-$) was cultured at 30°C with vigorous aeration in defined liquid medium with 20 mM acetate as the C source and 10 mM 6-hydroxynicotinate as the N source. After reaching stationary phase, cultures were centrifuged, and the supernatant was recovered and filtered. Conditioned medium was acidified to pH 1 with Dowex 50W-X8 resin (H$^+$ form), which removed 6-hydroxynicotinate, and twice

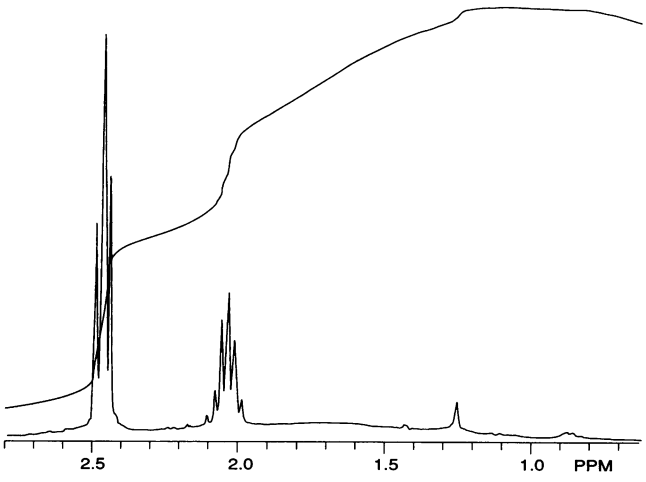

FIG. 2. Proton nuclear magnetic resonance spectrum of the crystalline compound purified from conditioned medium of strain 61510 culture. The sample was dissolved in chloroform. Except for the [^{1}H]chloroform solvent signal used to calibrate the scale, no down-field signals were present.

extracted (1:1 by volume) with ethyl acetate. The organic phase was collected and air dried overnight; this procedure removed residual acetic acid. The resulting white residue was resuspended in ethyl acetate, and insoluble material was removed by centrifugation. Upon addition of hexanes, crystals (12 mg) were obtained. The crystalline precipitate was collected by centrifugation, washed with hexane, and air dried. UV scanning spectrophotometry indicated that approximately 1% 6-hydroxynicotinate and THON remained in the resulting crystalline powder (data not presented). The nuclear magnetic resonance spectrum of the crystals was obtained and proved identical with that reported for glutaric acid (Fig. 2) (26). Infrared absorption spectra and mass spectra of the crystals were also obtained (data not presented); results were also consistent with those obtained for ACS reagent glutaric acid (Sigma).

By contrast, supernatant fractions from strain 61510 cultured on ammonium as the N source did not accumulate glutarate. Therefore, glutarate is produced by catabolism of 6-hydroxynicotinate.

Glutarate is transthioesterified to yield glutaryl-CoA, which is then oxidized by a respiratory dehydrogenase complex. Wild-type *A. caulinodans* was cultured in defined medium with several C sources; cell extracts were then assayed for respiratory GCADH activity, using glutaryl-CoA as an electron donor and ferricyanide as an electron acceptor (Materials and Methods); neither NAD^+ nor methylene blue served as an electron acceptor for glutaryl-CoA oxidation. GCADH activity was strongly induced when culture media were glutarate supplemented. With succinate as the C source, GCADH activity in wild-type cell extracts was 1/10 of that observed with glutarate as the C source (Table 3). Representative 6HN[C]$^-$ mutants were similarly tested for GCADH activity. When cultured in defined medium with acetate as the C source and 6-hydroxynicotinate as the N source, all strains showed GCADH activities at wild-type levels.

Glutarate-dependent thioacylation of coenzyme A (CoASH) was tested by a coupled assay. Although *Azorhizobium* cell-free extracts exhibited a short-lived, glutarate-dependent ferricyanide reduction, glutaryl-CoA-dependent ferricyanide reduction was sustained for more than 10 min at high rates. In glutarate-dependent assays, neither millimolar ATP addition nor millimolar CoASH addition nor both sustained ferricyanide reduction. However, millimolar succinyl-CoA stimulated ferricyanide reduction rates 50%, and high rates were sustained for at least 10 min (data not presented). Thus, *A. caulinodans* forms glutaryl-CoA by transthioesterification with succinyl-CoA. Representative *Azorhizobium* mutants were then assayed for SGCT activity as both glutarate- and succinyl-CoA-dependent ferricyanide reduction (Materials and Methods). In crude extracts of cells cultured on acetate and 6-hydroxynicotinate, strains 57100 (wild type), 61505, and 61606 showed comparable SGCT activities, whereas Glr$^-$ mutants 61510 and 61515 showed greatly reduced SGCT activities (Table 3).

TABLE 3. *Azorhizobium* GCADH and SGCT activities

Strain	Phenotype	Activity (nmol of ferricyanide reduced min^{-1} mg of protein^{-1})	
		GCADH	SGCT
57100 cultured in BASAL medium with:	Wild type		
20 mM succinate + 10 mM NH_4^+		4.3	ND
20 mM glutarate + 10 mM NH_4^+		47	34
20 mM acetate + 10 mM 6-hydroxynicotinate		21	14
61606[a]	6HN[N]$^+$ 6HN[C]$^-$	18	12
61505[a]	6HN[N]$^+$ 6HN[C]$^-$	13	7.0
61510[a]	6HN[N]$^+$ Glr$^-$	16	0.55
61515[a]	6HN[N]$^+$ Glr$^-$	17	0.38

[a] Cultured in basal medium with 20 mM acetate and 10 mM 6-hydroxynicotinate.

***Azorhizobium* mutants unable to use DL-β-hydroxybutyrate as a source of acetoacetyl-CoA cannot use 6-hydroxynicotinate as a C source.** When tested in liquid culture with defined medium, wild-type *A. caulinodans* grew on either D- or L-β-hydroxybutyrate as the sole C source. From Lineweaver-Burk plots, two NAD^+-dependent β-hydroxybutyrate dehydrogenase activities were kinetically distinguished in crude extracts of wild-type cells cultured with racemic DL-β-hydroxybutyrate as the C source; the two dehydrogenase activities specifically oxidized each of the two β-hydroxybutyrate enantiomers. As with the closely related *Alcaligenes eutrophus* (27), however, free D-β-hydroxybutyrate was oxidized to acetoacetate, while L-β-hydroxybutyrate was first thioacylated to form L-β-hydroxybutyryl-CoA, which was then oxidized to acetoacetyl-CoA (21). We thus inferred that *Azorhizobium* catabolism of racemic DL-β-hydroxybutyrate would converge at acetoacetyl-CoA, which is optically inactive. Similarly, acetoacetyl-CoA is a *Pseudomonas* glutarate catabolism intermediate (25). As discussed above, 21 independent *Azorhizobium* Vi mutants unable to utilize racemic DL-β-hydroxybutyrate as the sole C source (Table 1) were tested to delimit convergence of *Azorhizobium* nicotinate and DL-β-hydroxybutyrate pathways. All 21 mutants were Aac$^-$. Wild-type cells cultured on DL-β-hydroxybutyrate as the C source showed high levels of both acetoacetate:succinate-CoA transferase and β-ketothiolase activities (21). Because the wild type efficiently converted acetoacetate to ace-

TABLE 4. *Azorhizobium* dinitrogenase activities in culture

Strain	Phenotype	Acetylene reduction rate[a]		
		No supplement	Nicotinate	Valine
57100	Wild type	1.0	2.3	2.1
61302	6HN[N]$^-$	0.9	1.1	2.1
61606	6HN[N]$^+$ 6HN[C]$^-$	1.1	2.4	NT
61505	6HN[N]$^+$ 6HN[C]$^-$	1.2	2.4	NT
61510	6HN[N]$^+$ Glr$^-$	1.3	2.6	NT
63077	6HN[N]$^+$ βHB$^-$	1.0	2.3	NT
63083	6HN[N]$^+$ Aac$^-$	1.5	3.1	NT

[a] In NIF medium (4) supplemented with 16 μM nicotinate and 0.3 mM compounds indicated by the phenotypes. Values are normalized both for viable cell counts and for wild-type strain 57100 rates without supplementation. Data are mean values of three parallel experiments (standard errors of <15%). NT, not tested.

toacetyl-CoA, these 21 mutants must therefore be unable to use acetoacetyl-CoA. Only five mutants were able to grow on acetate as the sole C source (Table 1). Because the remaining 16 mutants grew normally on succinate as the sole C source, they were presumably affected in either or both glyoxylate bypass reactions catalyzed by isocitrate lyase and L-malate synthetase (19).

Most importantly, all 21 mutants were 6HN[C]$^-$ Glr$^-$ Nic[N]$^+$. Thus, acetoacetyl-CoA is indeed an intermediate in nicotinate catabolism, and as with conventional β-ketoacyl oxidations, *Azorhizobium* nicotinate catabolism terminates with conversion of crotonyl-CoA to acetoacetyl-CoA and then to two acetyl-CoA molecules. We may thus posit a complete pathway for *Azorhizobium* nicotinate catabolism (Fig. 1).

Nicotinate catabolism stimulates N_2 fixation by augmenting anabolic N pools. Under optimum physiological conditions, both nicotinate and 6-hydroxynicotinate stimulated wild-type *Azorhizobium* dinitrogenase rates threefold (Table 4). This stimulation was substrate concentration dependent and was optimal at 0.3 mM supplementation of either compound. As these physiological conditions entail steady-state N limitation, the wild type rapidly and exhaustively catabolizes nicotinate as a secondary N source (4, 11). To study physiological coupling of nicotinate catabolism and N_2 fixation, *Azorhizobium* 6HN[C]$^-$ mutants representative of each phenotypic subclass were assayed for dinitrogenase activity at various times after a physiological shift to N_2 fixation (Materials and Methods). Strain 61302, a nicotinate hydroxylase mutant in which N_2 fixation is not stimulated by added nicotinate (4), was included as a deficient experimental control. For both the wild-type and all 6HN[N]$^+$ 6HN[C]$^-$ mutants tested, when induction media were supplemented with 0.3 mM nicotinate, N_2 fixation rates were highly stimulated (Table 4). This mutant class seems to have an abortive nicotinate catabolism, which both produces ammonium for use as an N source and excretes C-skeleton intermediates, including glutarate. Consequently, ammonium is produced at rates sufficient for use as an anabolic N source but insufficient to create steady-state N excess, which would inhibit N_2 fixation.

Because this abortive nicotinate catabolism both stimulated *Azorhizobium* N_2 fixation and released limiting ammonium as an anabolic N source, these two processes might be physiologically coupled. Accordingly, we tested another *Azorhizobium* secondary N source for ability to stimulate N_2 fixation in culture. In wild-type defined liquid cultures, L-valine served satisfactorily as an N source but not as a C source. Therefore, the wild type and strain 61302 were cultured in defined medium with succinate as the C source, 0.3 mM L-valine as the N source, and limiting (16 μM) nicotinate as a vitamin. L-Valine mimicked nicotinate, stimulating N_2 fixation rates by both strains twofold (Table 4). Moreover, strain 57611, an ammonium assimilation mutant defective in L-glutamate synthase activity (5, 6), grew normally on L-valine as an N source. Hence, L-valine is transaminated, presumably by L-leucine aminotransferase, to directly yield L-glutamate, and not ammonium, as an anabolic N source. Thus, stimulation of N_2 fixation correlated with a general increase in steady-state anabolic N pools rather than with a specific increase in endogenous ammonium.

Therefore, nicotinate catabolism stimulates *Azorhizobium* N_2 fixation by augmenting anabolic N pools. When optimally fixing N_2 in culture with excess succinate as a C source, *A. caulinodans* catabolizes nicotinate at high rates yet remains physiologically N limited. As a consequence of nicotinate-released ammonium, *A. caulinodans* may sustain higher anabolic rates, and thus higher dinitrogenase biosynthetic rates, without suffering excess ammonium-mediated, feedback-inhibitory effects on N_2 fixation. By contrast, although *Azorhizobium* nicotinate catabolism presupposes both nicotinate- and glutaryl-CoA-driven respiratory oxidations, activity of neither respiratory chain correlates with N_2 fixation capability.

DISCUSSION

Azorhizobium nicotinate catabolism, at least up to ring breakage, is a chimera of two inferred pathways. As with all bacteria investigated (2, 13, 28), nicotinate is first oxidized to 6-hydroxynicotinate. Isolated nicotinate hydroxylases from *Pseudomonas* and *Bacillus* spp. both contain *c*-type cytochromes (15, 16). Similarly, one class of *Azorhizobium* Nic[N]$^-$ mutant lacks all *c*-type cytochromes (17, 18). Like its fellow aerobes, *A. caulinodans* channels electrons from the nicotinate hydroxylase complex through a cytochrome *c*-containing respiratory chain to O_2.

6-Hydroxynicotinate suffers different fates in different organisms. Surprisingly, as does *C. barkeri* (27), *A. caulinodans* next reduces 6-hydroxynicotinate to yield THON, which then undergoes hydrolytic ring breakage. In *C. barkeri*, the first intermediate identified subsequent to ring breakage was 2-methyleneglutarate. The inferred initial ring breakage product was 2-formylglutaryl-5-amide, produced by hydrolysis across the N-1–C-6 bond. Amide hydrolysis of 2-formylglutaryl-5-amide would then yield 2FG. In *C. barkeri*, nicotinate fermentation proceeds with the reduction of 2FG to yield 2-methyleneglutarate (28). In *A. caulinodans*, as reported here, [7-^{14}C]nicotinate was stoichiometrically converted to $^{14}CO_2$, and the first identified intermediate subsequent to ring breakage was glutarate. In contrast to the *C. barkeri* fermentative pathway, *A. caulinodans* oxidatively decarboxylates 2FG to yield glutarate. Indeed, this oxidative decarboxylation may be the only nicotinate catabolic reaction unique to *A. caulinodans*.

In patching together a nicotinate catabolic pathway with reactions taken from both anaerobes and aerobes, *A. caulinodans*, an obligate aerobe, avoids soluble dioxygenase reactions employed by fellow aerobes *Pseudomonas* and *Bacillus* spp. (2, 13). As *A. caulinodans* avidly catabolizes nicotinate during N_2 fixation, to which cytosolic O_2 would

pose a dire threat, its steady-state cytosolic O_2 level may be insufficient to facilitate reaction with soluble dioxygenases.

Uniquely, *A. caulinodans* converts nicotinate to glutarate, whose breakdown has also been studied in other aerobes. In *Pseudomonas fluorescens*, glutarate is thioesterified with free CoASH and, in an ATP-dependent reaction, yields glutaryl-CoA (24). By contrast, *A. caulinodans* employs glutarate and succinyl-CoA as cosubstrates in a thioacyltransferase (SGCT) reaction. Strains 61510 and 61515 showed sharply reduced SGCT activity, which correlated with a 6HN[C]$^-$ Glr$^-$ phenotype. The wild type also exhibited GCADH activity, which in *P. fluorescens* yields crotonyl-CoA (25). Indeed, *Azorhizobium* GCADH activity was induced by both glutarate and 6-hydroxynicotinate. Although not demonstrated, *Azorhizobium* GCADH activity presumably also yields crotonyl-CoA (Fig. 1). Then, as with archetypal acyl β oxidation, crotonyl-CoA hydrolase and L-β-hydroxybutyryl-CoA dehydrogenase, in concert, produce acetoacetyl-CoA, which then reacts with free CoASH via β-ketothiolase to yield two acetyl-CoA molecules. From mutant phenotypic and enzyme analyses reported here, this pathway operates similarly in *A. caulinodans*. Indeed, when growing on nicotinate as the sole C and N sources the wild type strongly induces glyoxylate bypass activity, indicative of steady-state acetyl-CoA excess (21).

If *A. caulinodans* were to produce acetyl-CoA from glutarate, then Aac$^-$ Ace$^+$ mutants would also be Glr$^-$. From a total of 33 6HN[N]$^+$ 6HN[C]$^-$ random insertion mutants, we subclassified sixteen Glr$^-$ Aac$^-$ mutants and five Glr$^-$ Aac$^-$ Ace$^+$ mutants. Therefore, *A. caulinodans* and *P. fluorescens* catabolize glutaryl-CoA similarly. In sum, *A. caulinodans* catabolizes nicotinate to yield ammonium, two CO_2 molecules, and two acetyl-CoA molecules.

Because it requires nicotinate for NAD^+ synthesis, and because under optimum N_2-fixing physiological conditions it rapidly catabolizes nicotinate as an N source, the wild-type *Azorhizobium* strain is not a true diazotroph (22). In *Azorhizobium* N_2-fixing chemostat cultures, less than 10% of the assimilated N culture yield was estimated as derived from nicotinate N; more than 90% was derived from fixed N_2 (3, 12). Therefore, N_2-fixing, N-limited continuous culture yields are only marginally affected by increased nicotinate, yet in batch culture, nicotinate is exhausted and NAD^+ becomes growth limiting (11). However, in both continuous and batch cultures, increased nicotinate indeed correlates with increased dinitrogenase activity (4, 12). By contrast, for either *Azorhizobium* Nic[N]$^-$ or 6HN[N]$^-$ mutants, increased nicotinate supplementation did not stimulate N_2 fixation. Hence, any physiological coupling with N_2 fixation involves nicotinate catabolism, not pyridine nucleotide biosynthesis. When nicotinate hydroxylase (Nic[N]$^-$ 6HN[N]$^+$) mutants are supplemented with increased 6-hydroxynicotinate, N_2 fixation is highly stimulated (4). Indeed, *Azorhizobium* 6HN[N]$^+$ 6HN[C]$^-$ mutants fix N_2 at wild-type rates, and N_2 fixation rates also respond to increased nicotinate.

If ammonium released by nicotinate catabolism stimulates N_2 fixation, would not ammonium added to N_2-fixing cultures effect the same outcome? To a very limited extent, yes. While 0.05 mM ammonium does slightly stimulate N_2-fixing batch cultures, at higher levels, ammonium strongly inhibits N_2 fixation (21). As with many diazotrophs, ammonium mediates transcriptional repression, covalent modification, allosteric inhibition, and product inhibition of dinitrogenase, all of which mitigate N_2 fixation (20, 23).

In *Azorhizobium* cultures, when nicotinate is further increased above 0.3 mM, N_2 fixation is inhibited (4). Therefore, 0.3 mM may be the exogenous nicotinate level that allows endogenous ammonium release at steady-state rates approximating those of prevailing ammonium assimilation. As a consequence, intracellular steady-state ammonium pools are low and insufficient to inhibit N_2 fixation. Nevertheless, under steady-state N limitation, higher ammonium assimilation rates imply higher dinitrogenase biosynthesis rates. When cultured with excess succinate as a C source, nicotinate, 6-hydroxynicotinate, or L-valine may be supplied as an N source at exogenous levels up to 0.3 mM and yet maintain endogenous steady-state N limitation. Still, anabolic rates of bacteria in N-limited cultures will respond to increases in endogenous anabolic N. In addition, after physiological shift of exponential-phase *Azorhizobium* cultures (or any other diazotrophic bacterial culture) to N-free medium, much net anabolism is dinitrogenase biosynthesis. Therefore, a subtle increase in steady-state anabolic N pools may profoundly affect N_2 fixation capacity.

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