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Rhizobium sp. Strain ORS571 Grows Synergistically on N_2 and Nicotinate as N Sources

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***Rhizobium* sp. strain ORS571 conducts synergistic, free-living N_2 fixation and nicotinate oxidation. Explicitly, ORS571 is able to fix N_2 aerobically because 6-OH-nicotinate acts as an intracellular O_2 sink. Because 6-OH-nicotinate oxidation is mandatory for aerobic, free-living N_2 fixation and because the synergistic processes yield ammonium from substrates (as the nitrogen source for growth), ORS571 is not a diazotroph.**

Rhizobium sp. strain ORS571 is unique among characterized rhizobia in undertaking both aerobic N_2 fixation ex planta as well as microaerobic N_2 fixation during symbiosis with *Sesbania rostrata* (4, 7, 9). As such, ORS571 appears to be a chimeric bacterium that exhibits properties of both diazotrophs and orthodox rhizobia. ORS571 N_2 fixation has been considered to operate in two metabolic modes. In free-living and early-nodulating bacteria, N_2 fixation and bacterial ammonium assimilation are coupled; when these bacteria persist as nongrowing bacteroids during the mature symbiosis, N_2 fixation and ammonium assimilation are decoupled (4).

ORS571 Nif^+ and Nif^- strains can be distinguished when tested for growth on solid, defined minimal medium (ORSNif [6]) without utilizable nitrogen sources and when maintained under a 98% N_2, 1% O_2, and 1%CO_2 atmosphere. N_2 fixation contributes to growth on solid media. However, ORS571 growth media must be supplemented with 7 μM nicotinate (1, 3, 4) because ORS571 cannot biosynthesize NAD^+ de novo (NAD^+ auxotroph).

Whereas ORS571 grew on solid ORSNif medium, it failed to grow on liquid ORSNif medium. Liquid ORSNif medium was inoculated with *Rhizobium* sp. strain ORS571 wild type, and the inoculated culture was divided in two. Both cultures were continuously sparged with gas mixtures containing excess N_2; one culture contained no added nitrogen source, the other contained 15 mM NH_4Cl. While the culture with ammonium grew normally, the culture without ammonium failed to grow (data not shown). Liquid ORSNif medium (6) contained excess succinate and D-glucose as energy sources and was supplemented with 7 μM nicotinate. Failure to grow in liquid ORSNif medium was not a function of exogenous O_2. Similar ORSNif liquid cultures were continuously sparged with gas mixtures containing greater than 98% N_2, 1% CO_2, and the following amounts of O_2: 21% (atmospheric), 3%, 1%, 0.2%, 0.02%, and 0.0015%. For 3 and 1% O_2, conventional gas flow meters were used to prepare gas mixtures; for the lower O_2 concentrations, prepared gas mixtures were purchased (Matheson Gas Co.). In no case did ORS571 growth ensue.

Although ORS571 failed to grow in liquid ORSNif medium containing limiting (7 μM) nicotinate, when excess nicotinate (14 mM) was supplied, normal growth was obtained under an atmosphere of 20% O_2–1% CO_2–balance argon. Moreover, added succinate, uniquely among carbon sources tested in these cultures, allowed the rhizobia to grow at comparable rates when either nicotinate or ammonium was used as the nitrogen source (data not shown). Two conclusions were drawn from these growth studies with ORS571. ORS571 can rapidly catabolize nicotinate in the presence of succinate, and a nicotinate supplement (7 μM) is rapidly exhausted when ORS571 is cultured in liquid ORSNif medium.

ORS571 mutants unable to utilize nicotinate as the sole nitrogen source (Nic^- mutants) were isolated by a vector-insertion (Vi) mutagenesis-cloning procedure described previously (6). Toward this end, random ORS571::Vi mutants carrying genomic insertions of plasmid pVP2021 were first isolated (6). Plasmid-genome cointegration is mediated by the $IS50_R$ element of Tn*5* (6). Subsequently, cultures of the random ORS571::Vi mutants were enriched for auxotrophs and then screened for mutants unable to use nicotinate as the sole nitrogen source (Nic::Vi mutants). Nine independent Nic::Vi mutants were obtained. All grew at rates indistinguishable from that of the wild-type on defined medium containing succinate as the carbon source and ammonium as the nitrogen source, as long as 7 μM nicotinate was included. None of the mutants exhibited pleiotropic effects on the ability to use a variety of nitrogen sources, such as nitrate or various amino acids. The inability of these Nic::Vi mutants to grow on nicotinate as the nitrogen source could not have resulted from the inability to take up nicotinate, because these mutants would otherwise have been deprived of NAD^+. By one hypothesis, wild-type ORS571 might be NAD^+ auxotrophic if intracellular nicotinate were constitutively catabolized to the extent that insufficient levels remained available for NAD^+ biosynthesis. However, because a block in the catabolism of nicotinate, as occurs in each of the Nic::Vi mutants, does not ameliorate the requirement for nicotinate (7 μM) supplementation, ORS571 is not NAD^+ auxotrophic by virtue of competitive nicotinate oxidation.

The ORS571 wild type and the Nic::Vi mutants were tested for growth in liquid, defined medium containing nicotinate, 6-OH-nicotinate, maleamate, and maleate-ammonium as sole carbon and nitrogen sources under an atmosphere of 20% O_2–1% CO_2–balance argon (Table 1). Of the four conditions, the ORS571 wild type grew on 6-OH-nicotinate only. While the ORS571 wild type failed to grow on medium containing nicotinate as sole carbon and nitrogen sources, it grew normally on succinate-nicotinate (Table 2). Although the ORS571 wild type grew on fumarate-ammonium medium, it failed to grow on fumarate-nicotinate medium (Table 2). Five of the nine Nic::Vi mutants, strains 61004, 61005, 61007, 61008, and 61009, grew on medium containing 6-OH-nicotinate as the sole carbon and nitrogen sources (Table 2). These were concluded to be nicotinate

TABLE 1. Bacterial strains used in this study

Strain	Phenotype[a]	Reference
Rhizobium sp.		
ORS571	Wild type	
Vi mutants (::pVP2021)	Km^r, Sm^r, Tc^r	6
60115	Nic^-, 6-OH-Nic^-, Nif^-	
60217	Nic^-, 6-OH-Nic^-, Nif^-	
60243	Nic^-, 6-OH-Nic^-, Nif^-	
61001	Nic^-, 6-OH-Nic^-, Nif^-	
61002	Nic^-, 6-OH-Nic^-, Nif^-	
61003	Nic^-, 6-OH-Nic^-, Nif^-	
61004	Nic^-, 6-OH-Nic^+	
61005	Nic^-, 6-OH-Nic^+	
61006	Nic^-, 6-OH-Nic^-, Nif^-	
61007	Nic^-, 6-OH-Nic^+	
61008	Nic^-, 6-OH-Nic^+	
61009	Nic^-, 6-OH-Nic^+	

[a] Abbreviations: Tc, tetracycline; Km, kanamycin; Sm, streptomycin; Nif, N_2 fixation; Nic, nicotinate oxidation; 6-OH-Nic, 6-OH-nicotinate oxidation.

hydroxylase mutants (Fig. 1). The inability of the ORS571 wild type to grow on maleate-ammonium or maleamate media might be attributed to the inability to import these *cis*-unsaturated derivatives.

The nicotinate catabolic pathway for ORS571 has not yet been rigorously determined, but it seems to be identical to that established for *Pseudomonas fluorescens* (Fig. 1 [1]), a related obligate aerobe. Compounds with the physical and spectral properties of 2,5-dihydroxypyridine, maleamate, maleate, and fumarate (1) have been isolated in significant amounts from ORS571 cultures growing on 6-OH-nicotinate as the sole carbon and nitrogen sources (data not shown).

If ORS571 nicotinate catabolism proceeds as shown in Fig. 1 (1), then the inability of ORS571 to grow on nicotinate as the sole carbon and nitrogen sources may be ascribed to the lack of an appropriate external reductant for nicotinate hydroxylase. Because succinate, but not fumarate, fulfills the metabolic requirement for such a reductant, it must act as the reductant (Fig. 1). Accordingly, because fumarate is the end product of nicotinate catabolism (1), the failure of ORS571 to grow on fumarate-nicotinate medium must result from succinate limitation. The ability of ORS571 to grow on 6-OH-nicotinate but not nicotinate as the sole carbon and nitrogen sources relates to the unambiguously oxidative metabolism of the former compound (members of the family *Rhizobiaceae* are obligate aerobes). Conversion of 1 mol of nicotinate to 0.5 mol of citrate (condensed in the trichloroacetic acid cycle operation) is net reductive metabolism; conversion of 1 mol of 6-OH-nicotinate to 0.5 mol of citrate is net oxidative metabolism.

The Nic::Vi mutants were tested for the ability to induce nitrogenase activity in liquid suspension cultures (in the presence of 7 μM nicotinate). Four of nine Nic::Vi mutants (strains 61001, 61002, 61003, and 61006) showed negligible acetylene reduction activities under all conditions tested; the remaining five mutants (strains 61004, 61005, 61007, 61008, and 61009) showed detectable acetylene reduction activities, however, that were greatly diminished when compared with that of the ORS571 wild type (Table 2).

When the nine Nic::Vi mutants were tested for growth on solid ORSNif medium (containing 7 μM nicotinate) two defective phenotypes, both distinct from that of the wild type, could be ascribed. Strains 61001, 61002, 61003, and 61006 (Table 1) formed pinpoint colonies, showed no growth enhancement when compared with similar plates cultured under argon instead of N_2, and were phenotypically identical to the ORS571 Nif^- control strain 60016 (6). These four strains thus possess Nif^- and 6-OH-$nicotinate^-$ phenotypes (Table 1).

Strains 61004, 61005, 61007, 61008, and 61009 formed gelatinous colonies on ORSNif medium, as did the wild type, but the mutant bacterial colonies were translucent and contained few cells, whereas wild-type colonies were opaque and filled with cells. These five Nic::Vi mutants were previously classed as nicotinate hydroxylase mutants (Table 1) because they grew on 6-OH-nicotinate as the sole carbon and nitrogen sources (Table 2). Indeed, when ORSNif solid medium was supplemented with 7 μM nicotinate and 7 μM 6-OH-nicotinate, these nicotinate hydroxylase mutants formed opaque colonies indistinguishable from those of the wild type. As noted above, these strains showed detectable acetylene reduction activities in tests with liquid cultures. These five mutants therefore constitute a second class of Nic^- mutants because they possess a conditional Nif^+ phenotype. If this interpretation is correct, then particulate nicotinate hydroxylase (1) activity is nonessential for N_2 fixation, whereas soluble 6-OH-nicotinate hydroxylations (or subsequent oxidations) are essential for N_2 fixation.

TABLE 2. Growth and N_2 fixation properties of ORS571 and Nic^- derivative strains

Strain	Growth in minimal, defined liquid medium containing the following as C and N sources[a]:								Nitrogenase activity (nmol C_2H_4)[b]
	Nic	S Nic	S NH_4^+	F Nic	F NH_4^+	6-OH-Nic	Mlm	Mle NH_4^+	
ORS571	−	+	+	−	+	+	−	−	3,400
60115	−	−	+	−	+	−	−	−	1,840
60217	−	−	+	−	+	−	−	−	520
60243	−	−	+	−	+	−	−	−	1,300
61001	−	−	+	−	+	−	−	−	130
61002	−	−	+	−	+	−	−	−	180
61003	−	−	+	−	+	−	−	−	<34
61004	−	−	+	−	+	+	−	−	1,140
61005	−	−	+	−	+	+	−	−	1,360
61006	−	−	+	−	+	−	−	−	160
61007	−	−	+	−	+	+	−	−	1,860
61008	−	−	+	−	+	+	−	−	1,270
61009	−	−	+	−	+	+	−	−	2,700

[a] Abbreviations: Nic, nicotinate; S, succinate; F, fumarate; 6-OH-Nic, 6-OH-nicotinate; Mlm, maleamate; Mle, maleate.
[b] Nanomoles of C_2H_2-dependent C_2H_4 produced per 6 h per 10^9 cells (5).

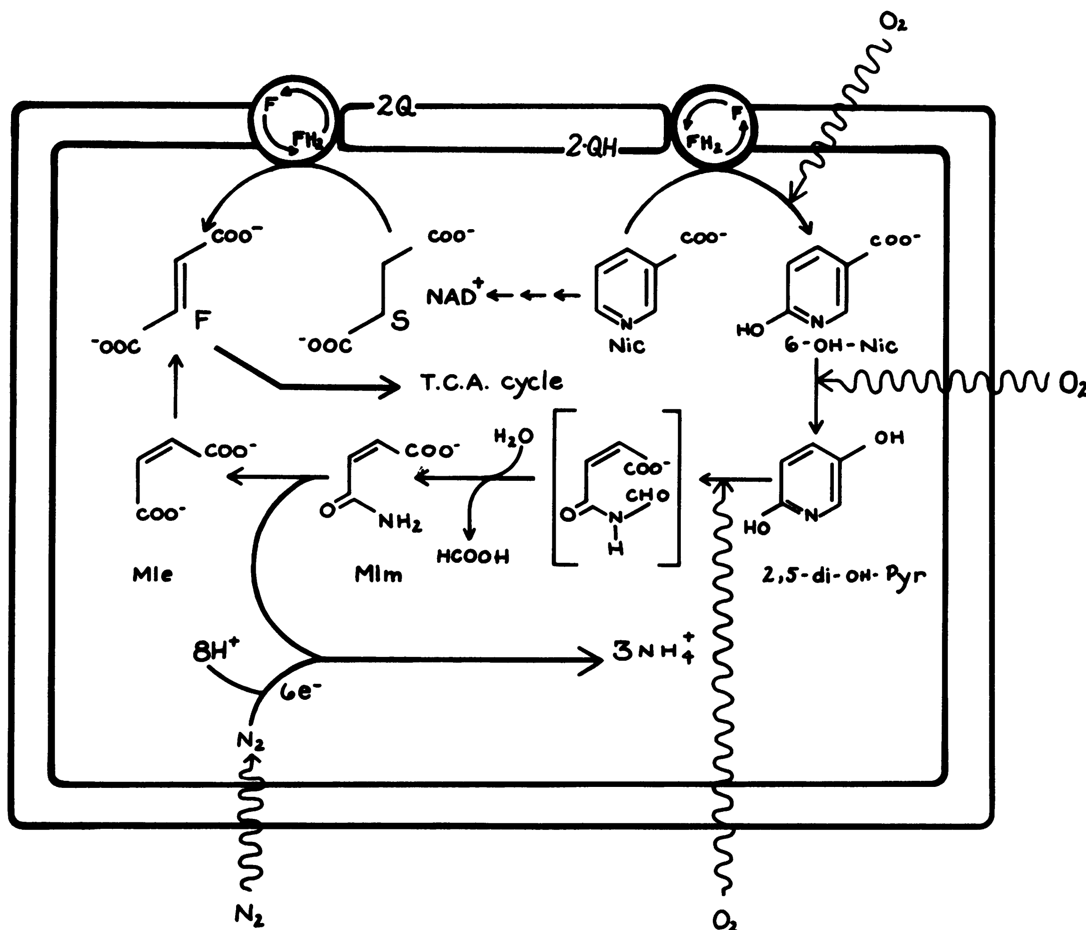

FIG. 1. Schematic model for the aerobic growth of *Rhizobium* sp. strain ORS571 during synergistic N_2 fixation and nicotinate oxidation. Nicotinate is substrate for both catabolic reactions and NAD^+ biosynthesis. Both nicotinate oxidation (1 mol of NH_4^+:1 mol of Nic catabolized) and N_2 (2 mol of NH_4^+:1 mol of N_2 reduced) yield ammonium as nitrogenous growth substituent. Fumarate, formate, and bicarbonate (from oxidation of 6-OH-nicotinate [6-OH-Nic]; not shown) are the carbon-containing end products of nicotinate catabolism. Abbreviations: F, flavin moiety; Q, membrane-localized quinone electron carrier; Mle, maleate; 2,5-di-OH-Pyr, 2,5-dihydroxypyridine; S, succinate; Mlm, maleamate; TCA, trichloroacetic acid.

Although their nodulation is delayed, all nine Nic::Vi mutants yield *S. rostrata* nodules. T symbiotic N_2 fixation activities of these nodules exactly parallel the free-living N_2 fixation activities of these nodules exactly parallel the free-living N_2 fixation activities of the Nic::Vi mutant rhizobia.

Approximately 100 independent Nif::Vi (not to be confused with Nic::Vi) mutants have been isolated, have yielded recombinant DNA clones, and have been physically mapped to the ORS571 genome (5). These Nif::Vi mutants have been screened for growth on ORSNif solid medium; mutant strains have been retained for further analyses only when they proved to be unambiguously negative in this growth test and also exhibited no pleiotropic effects on the utilization of diverse nitrogen sources. Surprisingly, three Nif::Vi mutants, strains 6G115, 60217, and 60243 (Table 1), yielded significant nitrogenase activities as determined by acetylene reduction (Table 2). When the Nic phenotypes of these Nif::Vi mutants were checked, none grew on either nicotinate or 6-OH-nicotinate as the nitrogen source (Table 2). Moreover, these mutants failed to grow on solid ORSNif medium supplemented with both 7 μM nicotinate and 7 μM 6-OH-nicotinate (data not shown). The insertion mutations in strains 60115, 60217, and 60243 are unlinked to the Nif loci (5). These mutants thus represent a third class of ORS571 Nic^- Nif^- strains.

ORS571 free-living and symbiotic N_2 fixation thus requires concomitant oxidative 6-OH-nicotinate catabolism. Both processes presumably yield ammonium as the nitrogenous growth substituent. Therefore, they are synergistic, because the presence of 6-OH-nicotinate allows the use of N_2, which is not otherwise utilizable and which is always present in excess. ORS571 is not able to grow on N_2 as the sole nitrogen source, as previously claimed (7). Explicitly, ORS571 conducts aerobic N_2 fixation when 6-OH-nicotinate is available to act as an intracellular O_2 sink. The soluble 6-OH-nicotinate hydroxylase and 2,5-dihydroxypyridine oxidase activities (1) scavenge, per mol of 6-OH-nicotinate oxidized, 2 mol of intracellular O_2. As a result of these reactions, the intracellular milieu might become sufficiently anaerobic so that nitrogenase-associated electron transfer reactions may proceed without interference from O_2.

Synergistic N_2 fixation and nicotinate catabolism both yield ammonium during aerobic metabolism (Fig. 1). Accordingly, the ORS571 ammonium assimilation pathway

functions like that of *Klebsiella pneumoniae*, a model free-living (facultative anaerobic) diazotroph, and not like that of orthodox rhizobia. The *Rhizobium* sp. strain ORS571 ammonium assimilation pathway is regulated in concert with N_2 fixation (4). On the other hand, ammonium assimilation in orthodox slow-growing rhizobia is decoupled from N_2 fixation both in culture and in planta (10). The orthodox slow-growing *Rhizobium* sp. strain 32H1, like many members of this group, can conduct free-living, microaerobic N_2 fixation. However, strain 32H1 does so in specialized, nongrowing cells; the ammonium produced is exported to the culture medium. Under certain conditions, this ammonium export allows syntrophic growth of heterogeneous cell populations (10). This orthodox rhizobial behavior correlates with that observed for symbiotic bacteroids, which do not grow and which export fixed ammonium to the plant cell cytoplasm for assimilation (3).

In this context, the aerobic, synergistic N_2 fixation and nicotinate catabolism of *Rhizobium* sp. strain ORS571 is idiosyncratic. The microecology of ORS571 is also unqiue. ORS571 forms nodules on root primordia borne on the stems of the tropical legume *S. rostrata* only when the host plant is systemically nitrogen limited. Because stem-infecting rhizobia do not have access to rhizosphere nitrogen sources, the major recourse is atmospheric N_2. Indeed, newly inoculated plants show detectable N_2 fixation activities within 48 h (unpublished data), which is 10 to 12 days before N_2 fixation activity commences in the mature nodules of other legumes. N_2 fixation therefore facilitates rhizobial proliferation during incipient nodulation. Necessarily, nicotinate or its derivatives, serving both as substrate for NAD^+ biosynthesis and catabolic oxidations, must be made available to the infecting rhizobia.

Nicotinate is a legume root growth factor; it is synthesized in *Pisum sativum* leaves and is systemically transported (2). Because the *S. rostrata* vascular tissue impinges on developing nodules, transported nicotinate is, in theory, available for nodular processes. In developing nodules, appropriate substrates are required for both the anabolic (NAD^+) and catabolic (6-OH-nicotinate) reactions during rhizobial proliferation. Nicotinate or its derivatives would play this dual metabolic role. The *P. sativum* vascular nicotinate supply has been estimated at 3 μM at steady state (2). Were this available in *S. rostrata*, ORS571 growth requirements would be satisfied.

Heretofore, the existence of a presumed diazotrophic *Rhizobium* spp., such as strain ORS571, has posed a more striking paradox than that of aerobic diazotrophy itself. If rhizobia grow while fixing N_2 during the early stages of nodule formation, why then do they not continue to proliferate in the host plant? Instead, *Rhizobium* sp. strain ORS571 becomes a docile endosymbiont, the proliferation of which ceases. By regulating the available nicotinate supply, the host plant might chart the course of the symbiosis. During early steps of nodule development, while rhizobia proliferate, nicotinate could be provided by the host plant; after nodule maturation, nicotinate could be withheld. Because leghemoglobin biosynthesis during nodule maturation leads to creation of an O_2-buffered, microaerobic environment, bacteroid N_2 fixation can be decoupled from nicotinate oxidation.

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