

Azorhizobium caulinodans Uses Both Cytochrome *bd* (Quinol) and Cytochrome *cbb*₃ (Cytochrome *c*) Terminal Oxidases for Symbiotic N₂ Fixation

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Azorhizobium caulinodans employs both cytochrome *bd* (*cytbd*; quinol oxidase) and *cytcbb*₃ (*cytc* oxidase) as terminal oxidases in environments with very low O₂ concentrations. To investigate physiological roles of these two terminal oxidases both in microaerobic culture and in symbiosis, knockout mutants were constructed. As evidenced by visible absorbance spectra taken from mutant bacteria carrying perfect gene replacements, both the *cytbd*⁻ and *cytcbb*₃⁻ mutations were null alleles. In aerobic culture under 2% O₂ atmosphere, *Azorhizobium cytbd*⁻ and *cytcbb*₃⁻ single mutants both fixed N₂ at 70 to 90% of wild-type rates; in root nodule symbiosis, both single mutants fixed N₂ at 50% of wild-type rates. In contrast, *Azorhizobium cytbd*⁻ *cytcbb*₃⁻ double mutants, which carry both null alleles, completely lacked symbiotic N₂ fixation activity. Therefore, both *Azorhizobium cytbd* and *cytcbb*₃ oxidases drive respiration in environments with nanomolar O₂ concentrations during symbiotic N₂ fixation. In culture under a 2% O₂ atmosphere, *Azorhizobium cytbd*⁻ *cytcbb*₃⁻ double mutants fixed N₂ at 70% of wild-type rates, presumably reflecting *cytaa*₃ and *cytbo* (and other) terminal oxidase activities. In microaerobic continuous cultures in rich medium, *Azorhizobium cytbd*⁻ and *cytcbb*₃⁻ single mutants were compared for their ability to deplete a limiting-O₂ sparge; *cytbd* oxidase activity maintained dissolved O₂ at 3.6 μM steady state, whereas *cytcbb*₃ oxidase activity depleted O₂ to submicromolar levels. Growth rates reflected this difference; *cytcbb*₃ oxidase activity disproportionately supported microaerobic growth. Paradoxically, in O₂-limited continuous culture, *Azorhizobium cytbd* oxidase is inactive below 3.6 μM dissolved O₂ whereas in *Sesbania rostrata* symbiotic nodules, in which physiological, dissolved O₂ is maintained at 10 to 20 nM, both *Azorhizobium cytbd* and *cytcbb*₃ seem to contribute equally as respiratory terminal oxidases.

Azorhizobium caulinodans, the sole member of its genus, uses at least five terminal oxidases, including cytochrome *aa*₃ (*cytaa*₃), *cytcbb*₃, and an alternative *a*-type cytochrome, which are specific for *cytc* as e⁻ donor, and *cytbo* and *cytbd*, which are specific for quinol as e⁻ donor. From spectroscopic measurements, in any given physiological O₂ environment, *A. caulinodans* uses multiple terminal oxidases (17, 25). From genetic analyses, null mutations in *Azorhizobium* terminal oxidase genes have little or no phenotypic consequence; therefore, these various terminal oxidases are somewhat degenerate (17, 19). *Azorhizobium* null mutants lacking either *cytaa*₃ or *cytbd* oxidase show little growth impairment; *cytaa*₃⁻ *cytbd*⁻ double mutants are still relatively healthy (17). Accordingly, we have sought to understand in more detail how *A. caulinodans* makes effective use of multiple terminal oxidases.

Azorhizobium fixes N₂ both in pure culture and in symbiosis with the host legume *Sesbania rostrata* (10). For these two disparate processes, optimal O₂ environments vary some 3 orders of magnitude. When fixing N₂ in culture, *Azorhizobium* prefers 10 μM dissolved O₂; when fixing N₂ in planta, dissolved O₂ is maintained at 10 nM by leghemoglobin buffering activity (5). Hypothetically, multiple terminal oxidases with wide-ranging kinetic constants, including both *K_m*(O₂) and *V_{max}*(O₂) values, confer on *Azorhizobium* the physiological versatility required to carry out N₂ fixation both in culture and in planta. Conceiv-

ably, multiple terminal oxidases (i) expand the physiological range of O₂ environments under which *Azorhizobium* might ably fix N₂ or (ii) improve efficiencies and/or rates of N₂ fixation under specific O₂ environments.

Among aerobic and microaerophilic diazotrophic bacteria which fix N₂ in specific, but quite different, O₂ environments, specific terminal oxidases are critically important. In the diazotrophs *Azotobacter vinelandii* (16) and *Klebsiella pneumoniae* (16, 24), *cytbd* oxidase is critically important; *cytbd* null mutants are unable to fix N₂. In the endosymbionts *Rhizobium meliloti* and *Bradyrhizobium japonicum*, *cytcbb*₃ oxidase is critically important; in both organisms, *cytcbb*₃ null mutants are unable to fix N₂ in symbiosis (4, 21). As further evidence of its physiological versatility, *Azorhizobium* single null mutants in either *cytbd* oxidase (17) or *cytcbb*₃ oxidase (19), while slightly impaired, remain able to fix N₂ both in pure culture and in symbiosis with the host legume *S. rostrata*.

As reported here, *Azorhizobium* strains carrying double null mutations in both *cytbd* and *cytcbb*₃ oxidase have now been constructed. While still able to grow and fix N₂ in aerobic culture, *Azorhizobium cytbd*⁻ *cytcbb*₃⁻ strains are completely unable to fix N₂ in symbiosis. Paradoxically, while both *cytbd* and *cytcbb*₃ oxidases function similarly in symbiosis, in microaerobic culture they have distinctive physiological roles. While *cytbd* oxidase is able to sustain growth and respiration at or above 3.6 μM dissolved O₂, *cytcbb*₃ oxidase does so at submicromolar levels of dissolved O₂.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Reference or source
<i>A. caulinodans</i>		
57100	ORSS571, wild type	10
64050	<i>cydAB</i> :: Ω , Sm ^r Sp ^r	17
64611 (57611)	Δ (<i>cytNO</i>)::Km ^r Nif ⁺ Fix ⁺	19
64612 (57612)	Δ (<i>cytNO</i>)::Km ^r , Nif ⁺ Fix ⁺	19
64620	<i>cydAB</i> :: Ω Δ (<i>cytNO</i>), Sm ^r Sp ^r Km ^r	This work
64621	<i>cydAB</i> :: Ω Δ (<i>cytNO</i>), Sm ^r Sp ^r Km ^r	This work
<i>E. coli</i> S17-1	MM294 Pro Thi <i>hsdR</i> [::RP4 Δ Tn1 <i>ter</i> ::Mu <i>npt</i> ::Tn7]	23
Recombinant plasmids		
pRS3010	pSUP202, 6.0-kbp <i>Bgl</i> II insert, <i>A. caulinodans</i> <i>cytN</i> ⁺ <i>O</i> ⁺ <i>Q</i> ⁺ <i>P</i> ⁺	19
pRS3014	pRS3010 Δ (<i>cytNO</i>), Ap ^r Km ^r Cm ^r	19
pRS3015	pRS3010 Δ (<i>cytNO</i>), Ap ^r Km ^r Cm ^r	19
pRS3016	pRS3010 Δ (<i>cytNO</i>), Ap ^r Km ^r Cm ^s Gm ^r	This work
pRS3017	pRS3010 Δ (<i>cytNO</i>), Ap ^r Km ^r Cm ^s Gm ^r	This work

^a Ap^r, apramycin resistance; Cm^r, chloramphenicol resistance; Cm^s, chloramphenicol sensitivity.

MATERIALS AND METHODS

Bacterial strains and recombinant plasmids. Several *A. caulinodans* and *Escherichia coli* strains used in these experiments have been previously described, and their culture methods have been detailed (Table 1). For *Azorhizobium* N₂ fixation-dependent growth tests, both rich (GYPC) and defined (NIF) minimal media (9) were used. For N₂ fixation activities, dinitrogenase assays, both with bacterial cultures and with nodulated plants, were performed as described previously (19). To avoid confusion with previously reported strains, *Azorhizobium* strains 57611 and 57612 (19) are here renamed, respectively, 64611 and 64612 (Table 1). With the recognition that it encodes *cytcbb*₃ oxidase (12, 13), the *Azorhizobium fixNOQP* operon has been renamed the *cytNOQP* operon.

Construction of *Azorhizobium* *cytbd*⁻ *cytcbb*₃⁻ double mutants. Recombinant plasmids pRS3014 and pRS3015 were constructed from pRS3010, which carries an *Azorhizobium* *cytNO* deletion allele, by removal of the 2.1-kbp *Xho*I fragment and insertion of a pUC4K *Sa*II fragment carrying the *nptIII* gene, which encodes kanamycin-neomycin phosphotransferase (19). To facilitate selection of double recombinants, the pUC1318 *Eco*RI fragment carrying the *gat* gene, which encodes gentamicin-apramycin acetyltransferase, was inserted into the *Eco*RI site of both pRS3014 and pRS3015. The resulting plasmids, pRS3016 and pRS3017, were used as gene donors for substitution by perfect gene replacement of the *cytNO* deletion (Δ *cytNO*) allele in strain 64050, which also carries a mutated *cydAB* allele, as follows. *E. coli* donor strains S17-1/pRS3016 and S17-1/pRS3017, isolated after plasmid transformations, were used as conjugal donors with the recipient *Azorhizobium* strain 64050, which carries a *cydAB*:: Ω allele, which confers both the streptomycin-resistant (Sm^r) and spectinomycin-resistant (Spr) phenotypes (17). Because donor plasmids cannot replicate in the recipient *Azorhizobium* strain (*cytN*⁺*O*⁺), Δ *cytNO* merodiploids were selected as triply resistant (kanamycin-resistant [K^r], gentamicin-resistant [G^r], and Sp^r transconjugants and were verified by genomic hybridization tests, with *cytNO* sequences as the DNA probes (19). Subsequently, haploid Δ *cytNO* derivatives were isolated after nonselective subculture of merodiploid transconjugants. Haploid *cytNO* recombinants were selected as Km^r Sp^r and were scored as gentamicin-sensitive (Gm^s) derivatives. Recombinants were haploid for the Δ *cytNO* allele as verified by genomic DNA hybridizations.

Visible absorbance spectra of membranes from *Azorhizobium* microaerobic cultures. *Azorhizobium* strains were cultured (15 liters) in GYPCS medium (8) at 30°C (Bio-Flow IV fermentor; New Brunswick Scientific). Culture start points were calibrated by visible light scattering (*A*₆₀₀ = 0.05), and cultures were maintained under a 0.5% O₂ and 99.5% N₂ sparge at a high gas flow (28 liters min⁻¹). Under this gas atmosphere and at this sparge rate, but in sterile medium, 100% saturation at 30°C equalled 6 μ M dissolved O₂ as measured potentiometrically by a gas-permeable electrode (Ingold). The zero scale was set by sparging with pure N₂ at similar rates and at this sparge rate, but in sterile medium, and O₂ saturation were monitored until cultures had reached cell densities sufficient for harvesting. Cultures were harvested with a DC10L cell concentrator (Amicon); cells were pelleted by centrifugation at 20,000 \times g for 10 min and washed with 40 mM phosphate buffer (pH 7.0). The cell paste (between 5 and 10 g [wet weight]) was resuspended in phosphate buffer (40 ml), and cells were disrupted by ultrasonication. Unbroken cells were pelleted by centrifugation (30,000 \times g for 25 min), and cell extracts were treated to yield solubilized membrane preparations, as previously described (17). Air oxidized, dithionite-reduced, and reduced-plus-CO visible absorbance spectra were obtained by wavelength scanning. Difference spectra were obtained by numerical subtraction and were numerically smoothed with a five-channel, binomial algorithm (17).

RESULTS

The *Azorhizobium* *cytbd* *cytcbb*₃ oxidase double mutant shows a complete loss of symbiotic N₂ fixation ability. *Azorhizobium* strains 64050 (*cytbd*⁻), 64611 (*cytcbb*₃⁻), and 64621 (*cytbd*⁻ *cytcbb*₃⁻) were tested for N₂ fixation activities both in culture and in symbiosis with the host legume *S. rostrata* (Table 2). As previously reported, dinitrogenase activities in pure culture of both *Azorhizobium* *cytbd*⁻ oxidase single null mutant 64050 (17) and *Azorhizobium* *cytcbb*₃⁻ oxidase single null mutant 57611 (19), here renamed 64611, were each only slightly impaired when compared with the wild-type parent. The same result was obtained when dinitrogenase activity was assayed in the symbiosis of the mutants with host legume *S. rostrata*; both single null mutants were only slightly impaired. In contrast, *S. rostrata* root nodules elicited by *Azorhizobium* *cytbd*⁻ *cytcbb*₃⁻ oxidase double mutant 64621 (see Materials and Methods) showed no detectable dinitrogenase activity (Table 2). Together, both the *cytbd* and *cytcbb*₃ oxidases therefore account for all *Azorhizobium* terminal oxidase activity in symbiotic nodules.

Azorhizobium *cytbd*⁻ *cytcbb*₃⁻ double mutant 64621 was also tested for N₂ fixation activity in culture under 2% O₂ atmosphere, which is optimal. In this physiological condition, the dissolved O₂ tension (10 μ M) is approximately 1,000-fold increased over that prevailing in symbiosis, a consequence of excess leghemoglobin (5). *Azorhizobium* strain 64621 remained able to fix N₂ in culture; dinitrogenase activities were approx-

TABLE 2. *Azorhizobium* dinitrogenase activities in culture and in symbiosis

Strain	Phenotype	Dinitrogenase activity in:	
		Pure culture ^a	Symbiosis ^b
57100	Wild type	30.8 \pm 4	0.46 \pm 0.2
64050	<i>cytbd</i> ⁻	28.1 \pm 1.4	0.17 \pm 0.1
64611	<i>cytcbb</i> ₃ ⁻	20.5 \pm 1	0.20 \pm 0.07
64612	<i>cytcbb</i> ₃ ⁻	22.2 \pm 1.3	0.20 \pm 0.07
64620	<i>cytbd</i> ⁻ <i>cytcbb</i> ₃ ⁻	12 \pm 1.7	<0.01
64621	<i>cytbd</i> ⁻ <i>cytcbb</i> ₃ ⁻	10.7 \pm 1	<0.01

^a Values are in nanomoles of C₂H₄ per minute per milligram of protein \pm standard deviations and are means of at least five independent assays.

^b Values are in nanomoles of C₂H₄ per minute per milligram of protein (fresh weight) \pm standard deviations and are means of four series of four root-nodulated plants.

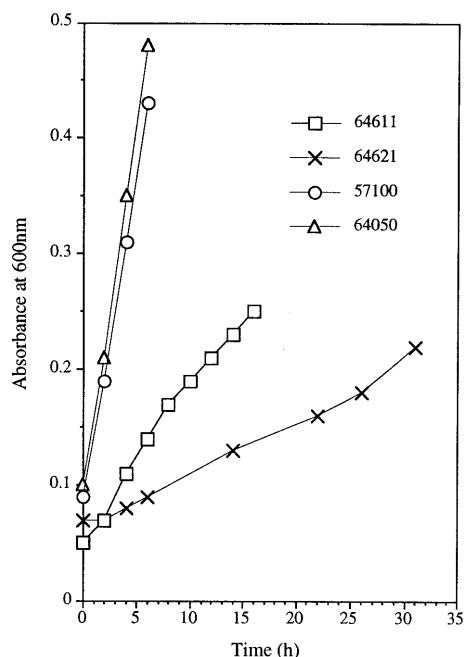


FIG. 1. Growth curves of *Azorhizobium* strains in liquid cultures under a 0.5% O₂ sparge.

imately 30% of wild-type levels (Table 2). This result was not unanticipated, since both *cytaa₃* oxidase and *cybo* oxidase are highly active under this condition (17).

***Azorhizobium cytcbb₃* oxidase is required for aerobic growth at very low O₂ concentrations.** The *Azorhizobium coxA* gene, which encodes subunit I of *cytaa₃* oxidase, is repressed at or below 0.5% O₂ atmosphere (17). Therefore, *Azorhizobium* strains were also tested for N₂ fixation in culture under a 0.1% O₂ atmosphere, at which both *cytaa₃* and *cybo* oxidase might be inactive. For plate growth experiments, *Azorhizobium* strains 57100 (wild type), 64050, 64611, and 64621 were inoculated on defined (NIF) solid medium to which (3 μM) nicotinate as a vitamin, but no other N source, was added (see Materials and Methods). Plates were incubated in sealed jars at 30°C under a continuous 0.1% O₂, 1.0% CO₂, and 98.9% N₂ sparge. After 7 days, both strain 57100 and strain 64050 yielded colonies similar in appearance; strains 64611 and 64621 yielded no colonies. Plates were similarly incubated a further 7 days and then reassessed; the results were unchanged. Therefore, both strain 64611 and strain 64621 were unable to grow on N₂ as the sole N source under 0.1% O₂. To test whether lack of growth reflected lack of N₂ fixation, all four strains were then cultured on rich (GYPCS) solid medium under a continuous 0.1% O₂ sparge. Similar results were obtained: strains 57100 and 64050 yielded colonies; strains 64611 and 64621 did not. Therefore, both strain 64611 and strain 64621 were unable to grow, in general, under a 0.1% O₂ atmosphere.

A similar experiment was then carried out with liquid batch cultures. Aerobic starter cultures of the four test strains in rich liquid medium were diluted ($A_{600} = 0.025$) and aerobically grown to 2×10^8 CFU ml⁻¹ ($A_{600} = 0.10$). At that point, liquid cultures were shifted to a 0.1% O₂, 1.0% CO₂, and 98.9% N₂ sparge. Both strain 57100 and strain 64050 continued to grow with little observable lag. However, as measured by light scattering (A_{600}), both strain 64611 and strain 64621 immediately stopped growing (data not presented). Therefore, *Azorhizobium* requires *cytcbb₃* oxidase activity for growth under 0.1%

O₂. As evidenced by its activity in symbiotic nodules, *cytbd* oxidase also functions as terminal oxidase in environments with nanomolar O₂ concentrations. However, *cytcbb₃* oxidase activity is required for growth on rich medium under 0.1% (micromolar) O₂, and any *cytbd* oxidase activity does not compensate for the loss of *cytcbb₃* oxidase activity.

Growth and dissolved O₂ concentrations of cultures established under a 0.5% O₂ sparge in rich medium. We sought to establish continuous cultures of strains 57100, 64050, 64611, and 64621 under a 0.5% O₂, 1% CO₂, and 98.5% N₂ sparge, the most limiting O₂ condition in which all four strains still grew. In sterile, rich GYPCS medium, the uninoculated, steady-state chemostat maintained 6 μM dissolved O₂ at saturation (see Materials and Methods). As measured by light scattering, under these limiting O₂ conditions, all four strains grew at linear, as opposed to exponential, rates (Fig. 1). During culture adaptations to changing O₂, dissolved O₂ was continuously monitored. Both *cytcbb₃*⁺ strains, 57100 and 64050, behaved similarly; 6 h after inoculation, cultures had exhausted dissolved O₂, which had stabilized at below-detectable (<0.5 μM) levels (Fig. 2). In contrast, *cytcbb₃*⁻ oxidase mutant 64611 grew at about one-third the wild-type rate. Some 10 h after inoculation, the dissolved O₂ environment of the 64611 culture had stabilized at 60% saturation (3.6 μM O₂). The *cytbd*⁻*cytcbb₃*⁻ oxidase double mutant 64621 grew at approximately 10% the wild-type rate, the lowest of all four strains. Some 20 h after inoculation, the dissolved O₂ environment of the 64621 culture had stabilized at 75% saturation (4.5 μM O₂). Therefore, both the *cytbd* and *cytcbb₃* terminal oxidases are active in rich medium culture under very low concentrations of O₂. Relative physiological roles of *Azorhizobium* terminal oxidases may then be inferred as follows. Aerobic terminal oxidases, which include *cytaa₃* and *cytbo*, etc., allow depletion of dissolved O₂ to 4.5 μM; *cytbd* oxidase activity allows dissolved O₂

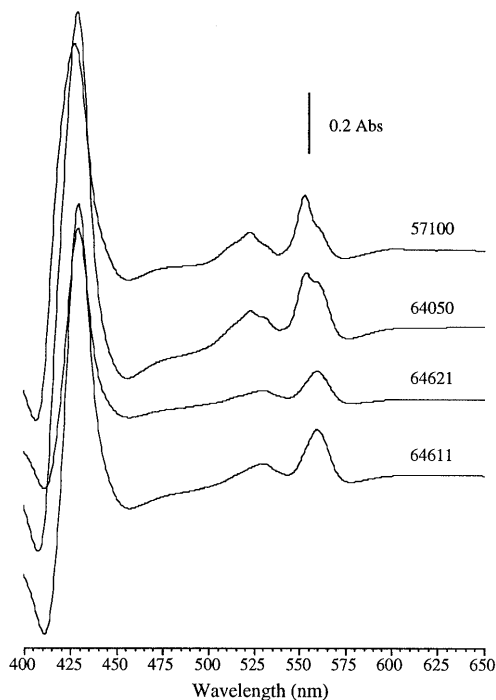


FIG. 2. Reduced-minus-oxidized light absorbance spectra of membranes from *Azorhizobium* cytochrome oxidase mutants cultured under a 0.5% O₂ sparge. Abs, absorbance.

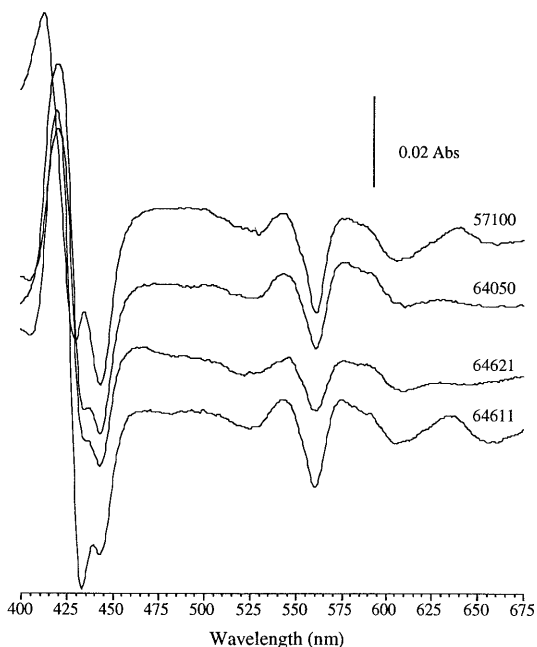


FIG. 3. Reduced-plus-CO-minus-reduced spectra of membranes from *Azorhizobium* cytochrome oxidase mutants cultured under a 0.5% O₂ sparge. Abs, absorbance.

depletion to 3.6 μ M; *cytcb*₃ oxidase activity allows depletion of dissolved O₂ to submicromolar levels.

Reduced-minus-oxidized light absorbance spectra of membranes from *Azorhizobium* cytochrome oxidase mutants cultured under a 0.5% O₂ sparge. To verify cytochrome oxidase phenotypes of *Azorhizobium* strains 57100, 64050, 64611, and 64621, chemostat cultures in rich medium were again established under a sparge with a 0.5% O₂ and 98.5% N₂ atmosphere (limiting O₂) at 30°C. As expected, cell membranes prepared from these O₂-limited cultures exhibited action spectra with greatly diminished levels of *cytaa*₃ oxidase activity (see Materials and Methods). However, reduced-minus-oxidized difference spectra of wild-type strain 57100 cell membranes did show a slight increase in *A*₆₀₅ (Fig. 2). Cell membranes from both strain 64611 and strain 64621 showed the characteristic loss of *cytc* at *A*₅₅₂, as previously noted for the *cytcb*₃⁻ oxidase single mutant (19). The *cytbd* oxidase signature, an absorbance peak at 630 nm, was absent in membranes prepared from strains 64050 and 64621. In addition, both strain 64611 and strain 64621 showed an absorbance peak in the Soret region at about 428 nm, a clear shift from the wild type (at 419 nm).

Reduced-plus-CO-minus-reduced spectra of membranes from *Azorhizobium* cytochrome oxidase mutants cultured under a 0.5% O₂ sparge. In the reduced-plus-CO-minus-reduced difference spectra, cell membranes from all four cultures showed the expected trough at 560 nm indicative of a *cytbo*-type oxidase (Fig. 3). In addition, all four strains exhibited the shoulder at 590 nm and the trough at 444 nm characteristic of a *cytaa*₃-type oxidase. In both wild-type 57100 and *cytcb*₃⁻ oxidase single mutant 64611, cell membranes exhibited the peak at about 640 nm characteristic of a *cytbd*-type oxidase. To various degrees, all four strains also exhibited another trough at between 430 and 435 nm. However, *cytbo*, *cytbd*, and *cytcb*₃ oxidases all exhibit troughs in this region, which complicates identification of any missing peaks. In summary, compared with those of the wild type, absorbance spectra were consistent

with the presumed null phenotypes of the studied terminal oxidase mutants.

Time course studies of *S. rostrata* stem nodule development: both the *cytbd* and *cytcb*₃ oxidases are similarly active in mature nodules. From results with cultures grown in rich medium with limiting O₂, *Azorhizobium* *cytbd* oxidase activity ceases below 3.6 μ M dissolved O₂. Yet, as inferred from genetic analyses, *cytbd* oxidase is active during symbiotic N₂ fixation, at which dissolved O₂ is 10 to 20 nM at steady state (5). Might *cytbd* oxidase function only relatively early in symbiotic nodule development, prior to leghemoglobin induction, when the O₂ concentrations remain relatively high (micromolar level and above)? To test this hypothesis, a temporal study of symbiotic stem nodule development was carried out. As a host, *S. rostrata*, which elicits determinate (developmentally synchronous) nodules, was used. Seedlings were germinated aseptically and grown (18) on sterile defined medium (14) under N limitation. Three-week-old seedlings, some 25 cm in height, were inoculated with *Azorhizobium* strain 57100, 64050, 64611, or 64621 between the first and second stem internodes; this procedure yields synchronized stem nodule development (9). Mature stem nodules were harvested 14 days postinoculation, sliced into two parts, and qualitatively inspected for leghemoglobin content by intense red coloration of nodule cortical tissue. By inspection, leghemoglobin was strongly induced 12 days after inoculation; as measured spectrophotometrically, leghemoglobin was fully induced 16 days after inoculation. Starting at day 16 and continuing daily to day 22 after inoculation, individual stem nodules were harvested and tested for N₂ fixation activity by acetylene reduction (see Materials and Methods). Wild-type 57100 yielded stem nodules with high N₂ fixation activities throughout this time course (Fig. 4). Stem nodules elicited by strains 64050, 64611, and 64621 were similarly harvested and analyzed; N₂ fixation rates were then normalized to those of wild-type nodules. Results corroborated those obtained with root nodules harvested at a single time point after inoculation (Table 2). Stem nodules

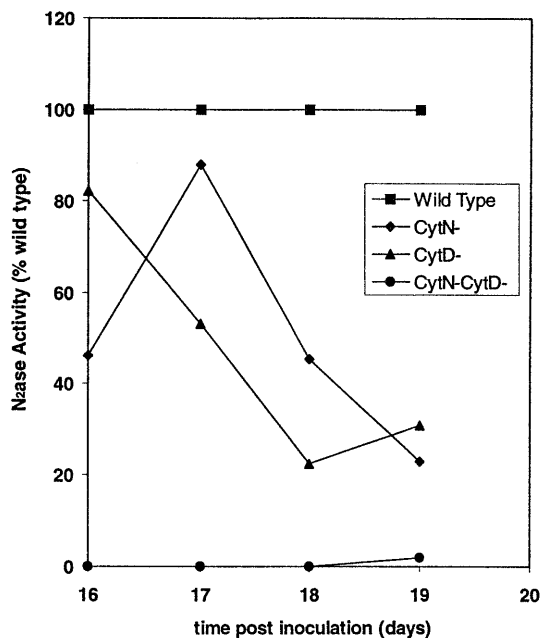


FIG. 4. Time course of N₂ fixation activities during *S. rostrata* stem nodule development. N₂ase, dinitrogenase.

elicited by strains 64050 and 64611 fixed N₂, but rates were low in comparison with that of the wild type. In mature nodules elicited by both 64050 and 64611, levels of N₂ fixation activities (relative to that of the wild type) similarly declined, probably as a consequence of progressive effects of impaired bacteroid oxidative phosphorylation (Fig. 4). Stem nodules elicited by 64621 showed essentially no detectable N₂ fixation activity throughout the experimental time course. By inference, in active stem nodules, symbiotic bacteroids of strain 64050 used *cytcbb*₃ as the sole terminal oxidase, whereas symbiotic bacteroids of strain 64611 used *cytbd* as the sole terminal oxidase. Therefore, even in stem nodules O₂ buffered with excess leghemoglobin and in which dissolved O₂ should have stabilized at 10 to 20 nM levels (5), both *cytbd* oxidase and *cytcbb*₃ oxidase remained similarly active.

DISCUSSION

In symbiotic legume nodules, leghemoglobins at high concentrations tightly bind, and thus buffer, physiological O₂. As a consequence, free dissolved O₂ drops to 10 to 20 nM at steady-state. (5). To drive oxidative phosphorylation, symbiotic bacteroids must use terminal oxidase(s) with extraordinarily low apparent *K_m*(O₂) values (3). In action spectra taken from free-living *B. japonicum* cultures, both *cytbo* and *cytaa*₃ terminal oxidases were highly active (2). Yet, in N₂-fixing symbiosis with *Glycine max* (soybean), *Bradyrhizobium* bacteroids, still capable of oxidative phosphorylation, showed neither *cytbo* nor *cytaa*₃ oxidase activity (1). In *B. japonicum*, an alternative *cytc*-dependent terminal oxidase, encoded by the *cycNOQP* operon, has been identified, cloned, and sequenced (21, 22). Indeed, neither *Rhizobium* (4) nor *Bradyrhizobium* (21) *cytN* mutants fix N₂ in symbiosis. The encoded terminal oxidase *cytcbb*₃ has been biochemically characterized for *Rhodobacter* spp. (12, 13), in which orthologous *cytNOQP* genes have been confirmed. In symbiotic *Rhizobium* and *Bradyrhizobium* bacteroids during N₂ fixation at nanomolar levels of dissolved O₂, *cytcbb*₃ oxidase activity alone allows rapid oxidative phosphorylation. Because *Bradyrhizobium* *cytc*₁⁻ insertion mutants also fail to fix N₂ in symbiosis (26), bacteroid oxidative phosphorylation requires a complete quinol→*cytc*₁→*cytc*→*cytcbb*₃→O₂ respiratory chain during symbiosis (6).

By contrast, *Azorhizobium* *cytN* single null mutants retain symbiotic N₂ fixation activity which, however, is somewhat diminished (19). Therefore, *cytcbb*₃ oxidase is active, but not so alone, under these conditions. Uniquely among the rhizobia, *Azorhizobium* also shows quinol-dependent *cytbd* oxidase activity (17). As with *cytcbb*₃⁻ mutants, *Azorhizobium* *cytbd*⁻ single null mutants fix N₂ in symbiosis, but at diminished rates in comparison with that of the wild type. Therefore, *cytbd* oxidase is also active in symbiotic nodules during N₂ fixation (17). Spectral absorbance studies confirmed the presumed null phenotypes of these mutants; neither *cytcbb*₃ nor *cytbd* activity was detected in respective single mutants (17, 19).

In this study, *Azorhizobium* *cytcbb*₃⁻ *cytbd*⁻ double mutants were constructed as recombinants carrying these two, single, null mutations. Again, as measured by spectral absorbance studies, double mutants completely lacked both *cytcbb*₃ and *cytbd* oxidase activities. When tested in symbiosis, double mutants completely lacked N₂ fixation activity. Therefore, during symbiosis, *Azorhizobium* uses both *cytcbb*₃ and *cytbd* terminal oxidases. Moreover, both *cytcbb*₃ and *cytbd* terminal oxidases seem to make similar, relative contributions to bacteroid respiration rates as inferred from N₂ fixation activities of whole nodules elicited by respective single mutants.

In culture, *Azorhizobium* uses at least five terminal oxidases

(17, 19). From physiological experiments with whole cells in culture, both *cytcbb*₃ and *cytbd* are collectively responsible for terminal oxidase activity at micromolar levels of dissolved O₂. However, *cytbd* oxidase activity ceases at or below 3.6 μM dissolved O₂, whereas *cytcbb*₃ oxidase remains active at submicromolar levels of dissolved O₂. During symbiosis with host *S. rostrata* plants, *Azorhizobium* bacteroids experience steady-state, dissolved O₂ levels in the 10 to 20 nM range (5). This poses an apparent paradox: why, then, is *cytbd* oxidase active during symbiosis? Indeed, we lack experimental results which might help reconcile this question.

Various aerobic, gram-negative bacteria, all members of the α-purple bacteria superfamily (27), drive aerobic respiration with quinol-dependent and/or *cytc*-dependent terminal oxidases. Several different classes of diazotrophic bacteria in this superfamily use only quinol-dependent terminal oxidases. Among these, the aerobic diazotrophs *Azotobacter chroococcum* and *Azotobacter vinelandii* employ both *cytbo* and *cytbd* as terminal oxidases (11, 28). In *Azotobacter vinelandii*, *cytbd* oxidase is absolutely required for aerobic N₂ fixation activity (16, 20). Likewise, in the facultative diazotroph *K. pneumoniae*, *cytbd* oxidase is required for microaerobic N₂ fixation (15, 24). In this sense there is a correlation with *Azorhizobium*, which, like all members of the family *Rhizobiaceae*, respire with both quinol-dependent and *cytc*-dependent terminal oxidases. Alone among the rhizobia, however, *Azorhizobium* both fixes N₂ at high rates in culture and exhibits *cytbd* oxidase activity. Yet, *cytbd* oxidase activity is not required for (relatively aerobic) *Azorhizobium* N₂ fixation activity in culture.

From comparative phylogenetic evidence, *cytc*-type oxidases seem relatively ancient. The relatively modern quinol oxidases might have their evolutionary origins in gram-positive bacteria. Subsequently, the quinol oxidases might have made their way to α-purple bacteria, a consequence of horizontal gene transfer events (7). In the relatively recent evolution of members of the family *Rhizobiaceae*, did *Azorhizobium* reacquire or simply maintain *cytbd* oxidase? In the former case, *Azorhizobium* *cytbd* oxidase activity might have conferred some symbiotic advantage, possibly to N₂ fixation itself. *Azorhizobium* has been isolated from the wild only from *S. rostrata* nodules. From comparative ultrastructures, *S. rostrata* elicits, both in stems and in roots, quite typical determinate nodules. Moreover, in comparison with that of many legumes, *S. rostrata* nodule physiology does not seem in any way unique. Therefore, exactly how the *Azorhizobium*-*Sesbania* symbiosis might uniquely benefit from *cytbd* oxidase activity remains to be understood.

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