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Azorhizobium caulinodans Uses Both Cytochrome *bd* (Quinol) and Cytochrome *cbb*$_3$ (Cytochrome *c*) Terminal Oxidases for Symbiotic N_2 Fixation

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Azorhizobium caulinodans **employs both cytochrome *bd* (cyt*bd*; quinol oxidase) and cyt*cbb*$_3$ (cyt*c* oxidase) as terminal oxidases in environments with very low O_2 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 cyt*bd*$^-$ and cyt*cbb*$_3^-$ mutations were null alleles. In aerobic culture under 2% O_2 atmosphere, *Azorhizobium* cyt*bd*$^-$ and cyt*cbb*$_3^-$ single mutants both fixed N_2 at 70 to 90% of wild-type rates; in root nodule symbiosis, both single mutants fixed N_2 at 50% of wild-type rates. In contrast, *Azorhizobium* cyt*bd*$^-$ cyt*cbb*$_3^-$ double mutants, which carry both null alleles, completely lacked symbiotic N_2 fixation activity. Therefore, both *Azorhizobium* cyt*bd* and cyt*cbb*$_3$ oxidases drive respiration in environments with nanomolar O_2 concentrations during symbiotic N_2 fixation. In culture under a 2% O_2 atmosphere, *Azorhizobium* cyt*bd*$^-$ cyt*cbb*$_3^-$ double mutants fixed N_2 at 70% of wild-type rates, presumably reflecting cyt*aa*$_3$ and cyt*bo* (and other) terminal oxidase activities. In microaerobic continuous cultures in rich medium, *Azorhizobium* cyt*bd*$^-$ and cyt*cbb*$_3^-$ single mutants were compared for their ability to deplete a limiting-O_2 sparge; cyt*bd* oxidase activity maintained dissolved O_2 at 3.6 μM steady state, whereas cyt*cbb*$_3$ oxidase activity depleted O_2 to submicromolar levels. Growth rates reflected this difference; cyt*cbb*$_3$ oxidase activity disproportionately supported microaerobic growth. Paradoxically, in O_2-limited continuous culture, *Azorhizobium* cyt*bd* oxidase is inactive below 3.6 μM dissolved O_2 whereas in *Sesbania rostrata* symbiotic nodules, in which physiological, dissolved O_2 is maintained at 10 to 20 nM, both *Azorhizobium* cyt*bd* and cyt*cbb*$_3$ 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*$_3$ (cyt*aa*$_3$), cyt*cbb*$_3$, and an alternative *a*-type cytochrome, which are specific for cyt*c* as e^- donor, and cyt*bo* and cyt*bd*, which are specific for quinol as e^- donor. From spectroscopic measurements, in any given physiological O_2 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 cyt*aa*$_3$ or cyt*bd* oxidase show little growth impairment; cyt*aa*$_3^-$ cyt*bd*$^-$ 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 N_2 both in pure culture and in symbiosis with the host legume *Sesbania rostrata* (10). For these two disparate processes, optimal O_2 environments vary some 3 orders of magnitude. When fixing N_2 in culture, *Azorhizobium* prefers 10 μM dissolved O_2; when fixing N_2 in planta, dissolved O_2 is maintained at 10 nM by leghemoglobin buffering activity (5). Hypothetically, multiple terminal oxidases with wide-ranging kinetic constants, including both $K_m(O_2)$ and $V_{max}(O_2)$ values, confer on *Azorhizobium* the physiological versatility required to carry out N_2 fixation both in culture and in planta. Conceivably, multiple terminal oxidases (i) expand the physiological range of O_2 environments under which *Azorhizobium* might ably fix N_2 or (ii) improve efficiencies and/or rates of N_2 fixation under specific O_2 environments.

Among aerobic and microaerophilic diazotrophic bacteria which fix N_2 in specific, but quite different, O_2 environments, specific terminal oxidases are critically important. In the diazotrophs *Azotobacter vinelandii* (16) and *Klebsiella pneumoniae* (16, 24), cyt*bd* oxidase is critically important; cyt*bd* null mutants are unable to fix N_2. In the endosymbionts *Rhizobium meliloti* and *Bradyrhizobium japonicum*, cyt*cbb*$_3$ oxidase is critically important; in both organisms, cyt*cbb*$_3$ null mutants are unable to fix N_2 in symbiosis (4, 21). As further evidence of its physiological versatility, *Azorhizobium* single null mutants in either cyt*bd* oxidase (17) or cyt*cbb*$_3$ oxidase (19), while slightly impaired, remain able to fix N_2 both in pure culture and in symbiosis with the host legume *S. rostrata.*

As reported here, *Azorhizobium* strains carrying double null mutations in both cyt*bd* and cyt*cbb*$_3$ oxidase have now been constructed. While still able to grow and fix N_2 in aerobic culture, *Azorhizobium* cyt*bd*$^-$ cyt*cbb*$_3^-$ strains are completely unable to fix N_2 in symbiosis. Paradoxically, while both cyt*bd* and cyt*cbb*$_3$ oxidases function similarly in symbiosis, in microaerobic culture they have distinctive physiological roles. While cyt*bd* oxidase is able to sustain growth and respiration at or above 3.6 μM dissolved O_2, cyt*cbb*$_3$ oxidase does so at submicromolar levels of dissolved O_2.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
A. caulinodans		
57100	ORS571, wild type	10
64050	*cydAB*::Ω, Sm^r Sp^r	17
64611 (57611)	Δ(*cytNO*)::Km^r $Nif^{\pm}$ $Fix^{\pm}$	19
64612 (57612)	Δ(*cytNO*)::Km^r, $Nif^{\pm}$ $Fix^{\pm}$	19
64620	*cydAB*::Ω Δ(*cytNO*), Sm^r Sp^r Km^r	This work
64621	*cydAB*::Ω Δ(*cytNO*), Sm^r Sp^r Km^r	This work
E. coli S17-1	MM294 Pro Thi *hsdR* [::RP4ΔTn*1* *tet*::Mu *npt*::Tn*7*]	23
Recombinant plasmids		
pRS3010	pSUP202, 6.0-kbp *Bgl*II insert, *A. caulinodans* $cytN^+O^+Q^+P^+$	19
pRS3014	pRS3010 Δ(*cytNO*), Ap^r Km^r Cm^r	19
pRS3015	pRS3010 Δ(*cytNO*), Ap^r Km^r Cm^r	19
pRS3016	pRS3010 Δ(*cytNO*), Ap^r Km^r Cm^s Gm^r	This work
pRS3017	pRS3010 Δ(*cytNO*), 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* N_2 fixation-dependent growth tests, both rich (GYPC) and defined (NIF) minimal media (9) were used. For N_2 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* cyt*bd*$^-$ 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 *Sal*I fragment carrying the *nptII* 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 (kanamicin-resistant [KnO], gentamicin-resistant [Gm^F], 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% O_2 and 99.5% N_2 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 μM dissolved O_2 as measured potentiometrically by a gas-permeable electrode (Ingold). The zero scale was set by sparging with pure N_2 at similar rates and at 30°C. Both visible light scattering (A_{600}) and O_2 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 × *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 × *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* cyt*bd* cytcbb_3 oxidase double mutant shows a complete loss of symbiotic N_2 fixation ability. *Azorhizobium* strains 64050 (cyt*bd*$^-$), 64611 (cytcbb_3^-), and 64621 (cyt*bd*$^-$ cytcbb_3^-) were tested for N_2 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* cyt*bd*$^-$ 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* cyt*bd*$^-$ cytcbb_3^- oxidase double mutant 64621 (see Materials and Methods) showed no detectable dinitrogenase activity (Table 2). Together, both the cyt*bd* and cytcbb_3 oxidases therefore account for all *Azorhizobium* terminal oxidase activity in symbiotic nodules.

Azorhizobium cyt*bd*$^-$ cytcbb_3^- double mutant 64621 was also tested for N_2 fixation activity in culture under 2% O_2 atmosphere, which is optimal. In this physiological condition, the dissolved O_2 tension (10 μM) is approximately 1,000-fold increased over that prevailing in symbiosis, a consequence of excess leghemoglobin (5). *Azorhizobium* strain 64621 remained able to fix N_2 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 ± 4	0.46 ± 0.2
64050	cyt*bd*$^-$	28.1 ± 1.4	0.17 ± 0.1
64611	cytcbb_3^-	20.5 ± 1	0.20 ± 0.07
64612	cytcbb_3^-	22.2 ± 1.3	0.20 ± 0.07
64620	cyt*bd*$^-$ cytcbb_3^-	12 ± 1.7	<0.01
64621	cyt*bd*$^-$ cytcbb_3^-	10.7 ± 1	<0.01

[a] Values are in nanomoles of C_2H_4 per minute per milligram of protein ± standard deviations and are means of at least five independent assays.

[b] Values are in nanomoles of C_2H_4 per minute per milligram of protein (fresh weight) ± standard deviations and are means of four series of four root-nodulated plants.

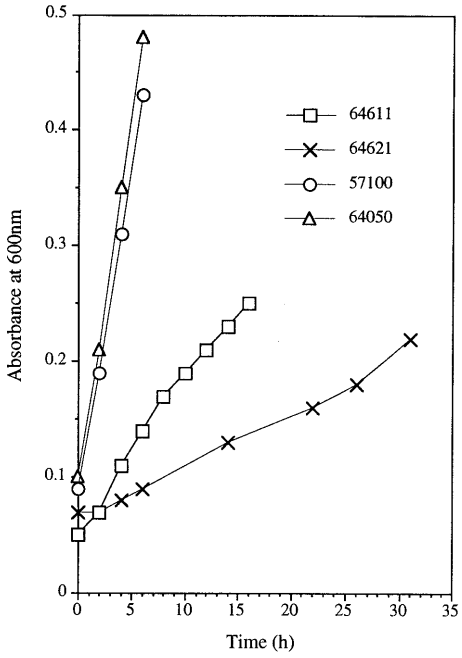

FIG. 1. Growth curves of *Azorhizobium* strains in liquid cultures under a 0.5% O_2 sparge.

imately 30% of wild-type levels (Table 2). This result was not unanticipated, since both cytaa_3 oxidase and cy*bo* oxidase are highly active under this condition (17).

***Azorhizobium* cytcbb_3 oxidase is required for aerobic growth at very low O_2 concentrations.** The *Azorhizobium coxA* gene, which encodes subunit I of cytaa_3 oxidase, is repressed at or below 0.5% O_2 atmosphere (17). Therefore, *Azorhizobium* strains were also tested for N_2 fixation in culture under a 0.1% O_2 atmosphere, at which both cytaa_3 and cyt*bo* 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% O_2, 1.0% CO_2, and 98.9% N_2 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 N_2 as the sole N source under 0.1% O_2. To test whether lack of growth reflected lack of N_2 fixation, all four strains were then cultured on rich (GYPCS) solid medium under a continuous 0.1% O_2 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% O_2 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% O_2, 1.0% CO_2, and 98.9% N_2 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 cytcbb_3 oxidase activity for growth under 0.1% O_2. As evidenced by its activity in symbiotic nodules, cyt*bd* oxidase also functions as terminal oxidase in environments with nanomolar O_2 concentrations. However, cytcbb_3 oxidase activity is required for growth on rich medium under 0.1% (micromolar) O_2, and any cyt*bd* oxidase activity does not compensate for the loss of cytcbb_3 oxidase activity.

Growth and dissolved O_2 concentrations of cultures established under a 0.5% O_2 sparge in rich medium. We sought to establish continuous cultures of strains 57100, 64050, 64611, and 64621 under a 0.5% O_2, 1% CO_2, and 98.5% N_2 sparge, the most limiting O_2 condition in which all four strains still grew. In sterile, rich GYPCS medium, the uninoculated, steady-state chemostat maintained 6 μM dissolved O_2 at saturation (see Materials and Methods). As measured by light scattering, under these limiting O_2 conditions, all four strains grew at linear, as opposed to exponential, rates (Fig. 1). During culture adaptations to changing O_2, dissolved O_2 was continuously monitored. Both cytcbb_3^+ strains, 57100 and 64050, behaved similarly; 6 h after inoculation, cultures had exhausted dissolved O_2, which had stabilized at below-detectable (<0.5 μM) levels (Fig. 2). In contrast, cytcbb_3^- oxidase mutant 64611 grew at about one-third the wild-type rate. Some 10 h after inoculation, the dissolved O_2 environment of the 64611 culture had stabilized at 60% saturation (3.6 μM O_2). The cytbd^- cytcbb_3^- 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 O_2 environment of the 64621 culture had stabilized at 75% saturation (4.5 μM O_2). Therefore, both the cyt*bd* and cytcbb_3 terminal oxidases are active in rich medium culture under very low concentrations of O_2. Relative physiological roles of *Azorhizobium* terminal oxidases may then be inferred as follows. Aerobic terminal oxidases, which include cytaa_3 and cyt*bo*, etc., allow depletion of dissolved O_2 to 4.5 μM; cyt*bd* oxidase activity allows dissolved O_2

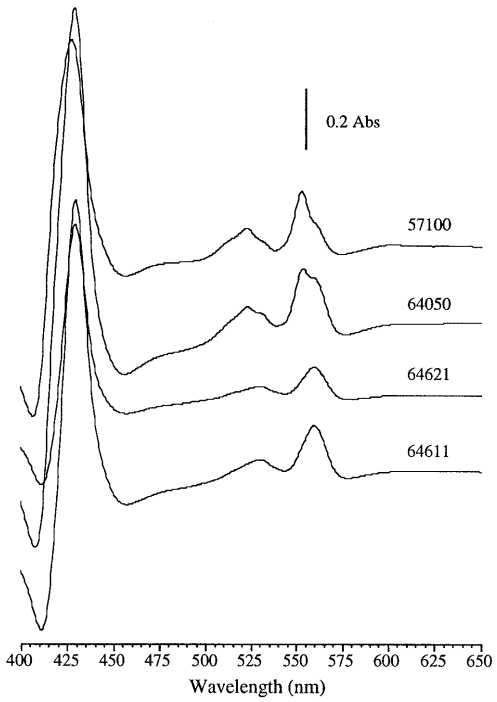

FIG. 2. Reduced-minus-oxidized light absorbance spectra of membranes from *Azorhizobium* cytochrome oxidase mutants cultured under a 0.5% O_2 sparge. Abs, absorbance.

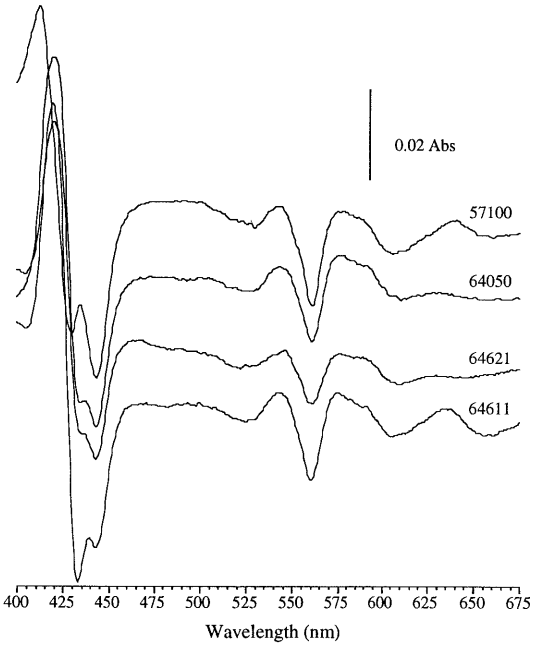

FIG. 3. Reduced-plus-CO-minus-reduced spectra of membranes from *Azorhizobium* cytochrome oxidase mutants cultured under a 0.5% O_2 sparge. Abs, absorbance.

depletion to 3.6 μM; cyt*cbb*$_3$ oxidase activity allows depletion of dissolved O_2 to submicromolar levels.

Reduced-minus-oxidized light absorbance spectra of membranes from *Azorhizobium* cytochrome oxidase mutants cultured under a 0.5% O_2 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% O_2 and 98.5% N_2 atmosphere (limiting O_2) at 30°C. As expected, cell membranes prepared from these O_2-limited cultures exhibited action spectra with greatly diminished levels of cyt*aa*$_3$ 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 cyt*c* at A_{552}, as previously noted for the cyt*cbb*$_3^-$ oxidase single mutant (19). The cyt*bd* 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% O_2 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 cyt*bo*-type oxidase (Fig. 3). In addition, all four strains exhibited the shoulder at 590 nm and the trough at 444 nm characteristic of a cyt*aa*$_3$-type oxidase. In both wild-type 57100 and cyt*cbb*$_3^-$ oxidase single mutant 64611, cell membranes exhibited the peak at about 640 nm characteristic of a cyt*bd*-type oxidase. To various degrees, all four strains also exhibited another trough at between 430 and 435 nm. However, cyt*bo*, cyt*bd*, and cyt*cbb*$_3$ 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 cyt*bd* and cyt*cbb*$_3$ oxidases are similarly active in mature nodules. From results with cultures grown in rich medium with limiting O_2, *Azorhizobium* cyt*bd* oxidase activity ceases below 3.6 μM dissolved O_2. Yet, as inferred from genetic analyses, cyt*bd* oxidase is active during symbiotic N_2 fixation, at which dissolved O_2 is 10 to 20 nM at steady state (5). Might cyt*bd* oxidase function only relatively early in symbiotic nodule development, prior to leghemoglobin induction, when the O_2 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 N_2 fixation activity by acetylene reduction (see Materials and Methods). Wild-type 57100 yielded stem nodules with high N_2 fixation activities throughout this time course (Fig. 4). Stem nodules elicited by strains 64050, 64611, and 64121 were similarly harvested and analyzed; N_2 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

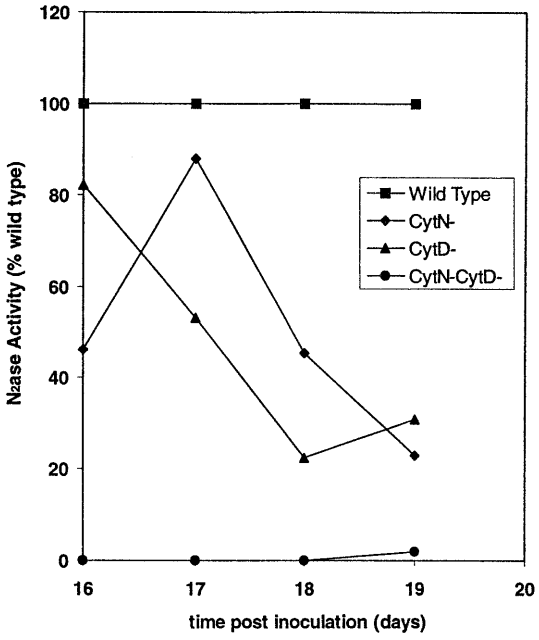

FIG. 4. Time course of N_2 fixation activities during *S. rostrata* stem nodule development. N2ase, dinitrogenase.

elicited by strains 64050 and 64611 fixed N_2, but rates were low in comparison with that of the wild type. In mature nodules elicited by both 64050 and 64611, levels of N_2 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 N_2 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 cyt*bd* as the sole terminal oxidase. Therefore, even in stem nodules O_2 buffered with excess leghemoglobin and in which dissolved O_2 should have stabilized at 10 to 20 nM levels (5), both cyt*bd* oxidase and cytcbb_3 oxidase remained similarly active.

DISCUSSION

In symbiotic legume nodules, leghemoglobins at high concentrations tightly bind, and thus buffer, physiological O_2. As a consequence, free dissolved O_2 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 $K_m(O_2)$ values (3). In action spectra taken from free-living *B. japonicum* cultures, both cyt*bo* and cytaa_3 terminal oxidases were highly active (2). Yet, in N_2-fixing symbiosis with *Glycine max* (soybean), *Bradyrhizobium* bacteroids, still capable of oxidative phosphorylation, showed neither cyt*bo* nor cytaa_3 oxidase activity (1). In *B. japonicum*, an alternative cyt*c*-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 N_2 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 N_2 fixation at nanomolar levels of dissolved O_2, cytcbb_3 oxidase activity alone allows rapid oxidative phosphorylation. Because *Bradyrhizobium* cytbc_1^- insertion mutants also fail to fix N_2 in symbiosis (26), bacteroid oxidative phosphorylation requires a complete quinol→cytbc_1→cyt*c*→cytcbb_3→O_2 respiratory chain during symbiosis (6).

By contrast, *Azorhizobium cytN* single null mutants retain symbiotic N_2 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 cyt*bd* oxidase activity (17). As with cytcbb_3^- mutants, *Azorhizobium* cytbd^- single null mutants fix N_2 in symbiosis, but at diminished rates in comparison with that of the wild type. Therefore, cyt*bd* oxidase is also active in symbiotic nodules during N_2 fixation (17). Spectral absorbance studies confirmed the presumed null phenotypes of these mutants; neither cytcbb_3 nor cyt*bd* 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 cyt*bd* oxidase activities. When tested in symbiosis, double mutants completely lacked N_2 fixation activity. Therefore, during symbiosis, *Azorhizobium* uses both cytcbb_3 and cyt*bd* terminal oxidases. Moreover, both cytcbb_3 and cyt*bd* terminal oxidases seem to make similar, relative contributions to bacteroid respiration rates as inferred from N_2 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 cyt*bd* are collectively responsible for terminal oxidase activity at micromolar levels of dissolved O_2. However, cyt*bd* oxidase activity ceases at or below 3.6 μM dissolved O_2, whereas cytcbb_3 oxidase remains active at submicromolar levels of dissolved O_2. During symbiosis with host *S. rostrata* plants, *Azorhizobium* bacteroids experience steady-state, dissolved O_2 levels in the 10 to 20 nM range (5). This poses an apparent paradox: why, then, is cyt*bd* 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 cyt*c*-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 cyt*bo* and cyt*bd* as terminal oxidases (11, 28). In *Azotobacter vinelandii*, cyt*bd* oxidase is absolutely required for aerobic N_2 fixation activity (16, 20). Likewise, in the facultative diazotroph *K. pneumoniae*, cyt*bd* oxidase is required for microaerobic N_2 fixation (15, 24). In this sense there is a correlation with *Azorhizobium*, which, like all members of the family *Rhizobiaceae*, respires with both quinol-dependent and cyt*c*-dependent terminal oxidases. Alone among the rhizobia, however, *Azorhizobium* both fixes N_2 at high rates in culture and exhibits cyt*bd* oxidase activity. Yet, cyt*bd* oxidase activity is not required for (relatively aerobic) *Azorhizobium* N_2 fixation activity in culture.

From comparative phylogenetic evidence, cyt*c*-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 cyt*bd* oxidase? In the former case, *Azorhizobium* cyt*bd* oxidase activity might have conferred some symbiotic advantage, possibly to N_2 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 cyt*bd* oxidase activity remains to be understood.

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