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Azorhizobium caulinodans Respires with at Least Four Terminal Oxidases

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In culture, *Azorhizobium caulinodans* used at least four terminal oxidases, cytochrome aa_3 (cytaa_3), cyt*d*, cyt*o*, and a second *a*-type cytochrome, which together mediated general, respiratory electron (e^-) transport to O_2. To genetically dissect physiological roles for these various terminal oxidases, corresponding *Azorhizobium* apocytochrome genes were cloned, and three cytaa_3 mutants, a cyt*d* mutant, and a cytaa_3, cyt*d* double mutant were constructed by reverse genetics. These cytochrome oxidase mutants were tested for growth, oxidase activities, and N_2 fixation properties both in culture and in symbiosis with the host plant *Sesbania rostrata*. The cytaa_3 mutants grew normally, fixed N_2 normally, and remained fully able to oxidize general respiratory e^- donors (NADH, succinate) which utilize a cyt*c*-dependent oxidase. By difference spectroscopy, a second, *a*-type cytochrome was detected in the cytaa_3 mutants. This alternative *a*-type cytochrome (A_{max} = 610 nm) was also present in the wild type but was masked by bona fide cytaa_3 (A_{max} = 605 nm). In late exponential-phase cultures, the cyt*aa*3 mutants induced a new, membrane-bound, CO-binding cytc_{550}, which also might serve as a cyt*c* oxidase (a fifth terminal oxidase). The cloned *Azorhizobium* cytaa_3 genes were strongly expressed during exponential growth but were deactivated prior to onset of stationary phase. *Azorhizobium* cyt*d* mutants showed 40% lower N_2 fixation rates in culture and in planta, but aerobic growth rates were wild type. The cytaa_3, cyt*d* double mutant showed 70% lower N_2 fixation rates in planta. Pleiotropic cyt*c* mutants were isolated by screening for strains unable to use *N,N,N',N'*-tetramethyl-*p*-phenylenediamine as a respiratory e^- donor. These mutants synthesized no detectable cyt*c*, excreted coproporphyrin, grew normally in aerobic minimal medium, grew poorly in rich medium, and fixed N_2 poorly both in culture and in planta. Therefore, while aerobic growth was sustained by quinol oxidases alone, N_2 fixation required cyt*c* oxidase activities. Assuming that the terminal oxidases function as do their homologs in other bacteria, *Azorhizobium* respiration simultaneously employs both quinol and cyt*c* oxidases. Because *Azorhizobium* terminal oxidase mutants were able to reformulate their terminal oxidase mix and grow more or less normally in aerobic culture, these terminal oxidases are somewhat degenerate. Its extensive terminal oxidase repertoire might allow *Azorhizobium* spp. to flourish in wide-ranging O_2 environments.

As biological N_2 fixation is extremely O_2 sensitive, N_2-fixing organisms have evolved O_2 detoxification and/or avoidance mechanisms. *Azotobacter* spp., free-living aerobes, elaborate cells with very high total membrane surface area and, consequently, very high concentrations of respiratory electron (e^-) transport complexes. For respiration, *Azotobacter* spp. employ cytochrome *d* (cyt*d*) and cytochrome *o* (cyt*o*) quinol oxidases but not cyt*c* oxidases (20, 44). To fix N_2 in air, *Azotobacter* spp. require cyt*d* oxidase (22), which might also mitigate oxidative stress (19, 22, 38). The physiological importance of cyt*o* remains unclear. *Klebsiella pneumoniae*, a facultative anaerobe, fixes N_2 in microaerobiosis and also employs a cyt*d* quinol oxidase (19, 38). Symbiotic N_2-fixing rhizobia, obligate aerobes, exhibit multiple cytochrome oxidases in culture and in planta. In culture, respiring rhizobia show branched e^- transport, which terminates at several oxidases. Common to one branch is a cyt*c* oxidase, usually cytaa_3, whereas other branches use quinol oxidases, usually cyt*o* (10, 31). Considering ATP yield, the cyt*c* oxidases are more efficient; i.e., they generate a larger proton motive force per e^- equivalent transported compared with the quinol oxidases.

In symbiotic legume nodules, rhizobia are buffered in situ against free O_2 by leghemoglobins, plant hemoproteins which tightly bind O_2 (3). When either growing as bacteria in culture or fixing N_2 as bacteroids in planta, rhizobia use different sets of terminal oxidases. Indeed, the pO_2 in these diverse environments differs by several orders of magnitude. In legume nodules, free O_2 is maintained at ~10 nM by high leghemoglobin levels. In order to drive oxidative phosphorylation, bacteroids must use terminal oxidases with exceedingly high O_2 affinities (3). In soybean nodules, *Bradyrhizobium japonicum* bacteroids lose all free-living terminal oxidases and express completely new cytochromes (3, 43). Whereas the *Bradyrhizobium* cytbc_1 complex is essential for symbiotic N_2 fixation, cytaa_3 oxidase is not (7, 41). The *Bradyrhizobium* bacteroid terminal oxidase has been identified and is a heme/copper cytochrome *c* oxidase (32).

Azorhizobium caulinodans, a composite of both diazotrophic bacteria and symbiotic rhizobia, avidly fixes N_2 both in culture and in planta (16). For these two disparate N_2 fixation processes, dissolved O_2 optima vary some 3 orders of magnitude: 10 μM in culture versus 10 nM in planta (5, 9). By spectrophotometry, *Azorhizobium* continuous cultures actively fixing N_2 show cyt*o*, cyt*d*, and cytaa_3 activities (40). To analyze their physiological roles, we here report the construction of *Azorhizobium* cytochrome oxidases by reverse genetics. *Azorhizobium* genes encoding apocytaa_3 and apocyt*d* were cloned and mutated by DNA insertion, and strains carrying these mutant alleles were constructed as perfect gene replacements. By conventional forward genetics, pleiotropic *Azorhizobium* mutants lacking all spectrophotometrically detectable *c*-type cytochromes were also isolated. Pleiotropic cyt*c* mutants both grew

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and fixed N_2 poorly. In contrast, by several criteria, the cytaa_3 and cyt*d* oxidase mutants remained able to grow and fix N_2 at more or less wild-type rates. From visible light absorbance difference spectra taken under a variety of physiological conditions, the *Azorhizobium* wild type used four terminal oxidases to drive general, respiratory e^- transport. Thus, the physiological roles of these terminal oxidases are somewhat redundant. This broad repertoire of terminal oxidases might allow *Azorhizobium* spp. to exploit a broad range of O_2 environments.

MATERIALS AND METHODS

Bacterial strains, mutant isolations, and growth media. *A. caulinodans* wild-type strain ORS571 (16) was subjected to vector insertion mutagenesis with plasmid pVP2021 as previously described (15). The random insertion library so obtained was screened for mutants unable to oxidize the artificial e^- donor tetramethyl-*p*-phenylenediamine (TMPD). Candidate colonies on agar plates were transferred to sterile Whatman no. 1 filters, which were then soaked 10 min in a 1% TMPD–0.1% ascorbic acid solution. Strains which failed to show diagnostic blue spots on filters were purified, retested, and screened for additional phenotypes (see text). *Azorhizobium* strains were cultured in either rich (GYPCS [9]) or basal (9) minimal defined medium with 0.2% dipotassium succinate as the C source and 10 mM either ammonium or nicotinate as the N source. Defined (MM) medium consisted of basal salts plus 0.2% succinate, 0.2% D-glucose, 0.1% L-glutamate, and 10 mM ammonium sulfate.

Molecular cloning and DNA physical mapping. *B. japonicum coxA* coding sequences were isolated from plasmid pCA1 as a 1.2-kbp *Eco*RI DNA fragment (Table 1). *Azotobacter vinelandii cydAB* coding sequences were isolated as a 3.0-kbp *Sac*I-*Eco*RI DNA fragment of plasmid pMH3 (Table 1). Both purified DNA fragments were used as DNA hybridization probes against an *Azorhizobium* genomic library constructed in λEMBL3 (15) by standard plaque hybridization techniques (35); *Escherichia coli* BNN45 served as the λ host. Phage λ DNA was purified as described previously (35), and *coxA* or *cydAB* homologous regions were physically mapped by endonuclease analysis and Southern hybridization (35). λ DNA fragments flanking identified homologous loci were then subcloned (Table 1) by standard techniques (35).

Visible light difference spectrophotometry. Whole cells and cell membrane suspensions were analyzed by spectrophotometry (Aminco DW-2a spectrophotometer) with 1-nm band width and 0.3-s response time; spectra were recorded at 0.5 nm s^{-1}. Whole cells, cultured to stationary phase in MM medium, were concentrated to 40% (wt/vol) in 15 mM potassium phosphate, pH 7.0 (2XP buffer). To take reduced-minus-oxidized difference spectra, cell suspensions in sample cuvettes were first treated with 1 mg of sodium dithionite and, in reference cuvettes, with 1 mg of ammonium persulfate. Because random light scattering from opaque samples limited whole-cell spectra, CO-bound reduced-minus-reduced difference spectra were recorded from isolated cell membranes. Cells were cultured to early stationary phase in MM medium. Cultures (2 liters) were harvested by centrifugation and resuspended (10 ml) in 2XP buffer. Cells were lysed by three passages through a French pressure cell at 2,000 external lb/in^2. Unbroken cells and some cell wall components were removed by centrifugation for 30 min at 12,000 × *g*. Cell membranes were pelleted by centrifugation for 2 h at 200,000 × *g*. Membranes were resuspended (1 ml) in 2XP buffer. Visible absorbance spectra were obtained from dithionite-reduced samples against a water blank and then stored in digitized form (Nicolet 1180 computer). Sample cuvettes were then sealed with rubber stoppers and flushed for 5 to 10 min with 100% CO, and a second spectrum was then obtained as described above. To yield the difference spectrum, these two spectra were numerically subtracted. Because cytaa_3 was poorly expressed in late exponential-phase cultures (see Results), early exponential-phase cultures in larger volumes were then prepared. Large-volume (15-liter) cultures in GYPCS medium were grown in a New Brunswick Bioflow IV fermenter, harvested at A_{600} = 0.12 (~2 × 10^8 cells ml^{-1}), concentrated by micropore filtration (Amicon filter), and resuspended in 40 ml of 2XP buffer. The cells were broken by ultrasonication, and the membranes were separated as described above. To clarify cell fractions for spectroscopy, membranes were solubilized in 0.8% dodecylmaltoside. Insoluble cell wall material was pelleted by centrifugation for 1.5 h at 200,000 × *g*. Samples were oxidized by flushing the headspace of sealed cuvettes with 100% O_2, were reduced with sodium dithionite, and were flushed with 100% CO. Primary spectra were then taken with a Jasco V-560 spectrophotometer, and the appropriate difference spectra were obtained by numerical subtraction.

β-Glucuronidase activity. *Azorhizobium coxA*::*uidA* strain 64003 was cultured (1 liter) to early exponential phase at 30°C in MM medium. Samples (40 ml) were removed at indicated times, culture turbidity (A_{600}) was measured, and cells were

TABLE 1. Bacterial strains, phages, and plasmids used

Strain, phage, or plasmid	Genotype or phenotype	Source or reference
E. coli		
HB101	*pro leu lacYsupE4 rspL endA hsdR hsdM* Sm^r	H. W. Boyer
DH5α	DU169 *recA1 endA1 supE44 thi-1 gyrA96 relA1* (φ80*lacZ*)	LifeTech Inc.
BNN45	*thi met hsdR hsdM supE4 supF*	R. W. Davis
A. caulinodans		
57100	Wild type	B. Dreyfus
64001	*coxA*::Tn*5uidA7* Km^r	This work
64002	*coxA*::Tn*5uidA7* Km^r	This work
64003	*coxA*::Tn*5uidA7* Km^r	This work
64050	*cydAB*::Ω Sm^r Sp^r	This work
64100	*coxA*::Tn*5uidA7 cydAB*::Ω Km^r Sm^r Sp^r	This work
61207	$cytc^-$ Glr^- $Nic[N]^-$ $TMPD^-$	This work
Phages		
λCya1	λEMBL3 *coxA*$^+$	This work
λCyd1	λEMBL3 *cydA*$^+$ *B*$^+$	This work
λCyd2	λEMBL3 *cydA*$^+$*B*$^+$	This work
λCyd3	λEMBL3 *cydA*$^+$ *B*$^+$	This work
λTn*5uidA*	λ429::Tn*5uidA7*	36
Plasmids		
pRK2073	ColE1 Tra^+ Sp^r	13
pSUP202	ColE1 Mob^+ Tc^r Ap^r Cm^r	37
pVP2021	pSUP202::Tn*5* Km^r Tc^r Sm^r Ap^r Cm^r Tra^+	16
pKAR1	pSUP202 *coxA*$^+$ Ap^r Tc^r	This work
pKARG1	pKAR1::Tn*5uidA7* Ap^r Tc^r Km^r	This work
pKARG2	pKAR1::Tn*5uidA7* Ap^r Tc^r Km^r	This work
pKARG3	pKAR1::Tn*5uidA7* Ap^r Tc^r Km^r	This work
pCDN9	pSUP202 *cydA*$^+$*B*$^+$, Ap^r Tc^r	This work
pCDN9SS	pCDN9::Ω Ap Tc^r Sm^r Sp^r	This work
pCAP66	pSUP202::Ω Ap^r Sm^r Sp^r	6
pCA1	pLAFR1::*coxA*$^+$ Tra^+ Tc^r	30
pMH3	ColE1 *cydA*$^+$*B*$^+$ Ap^r	26
pSB482	ColE1::Tn*5uidA7* Km^r Ap^r	36

collected by centrifugation at 4°C. Cell pellets were resuspended (minimal volume) in GUS extraction buffer (21) and lysed by ultrasonication. Unbroken cells were removed by centrifugation, and cell extracts were then assayed for β-glucuronidase activity with 4-methylumbelliferyl-β-D-glucuronide as the fluorogenic substrate (21). Total protein in cell extracts was measured by the method of Bradford (4).

Dinitrogenase activity. N_2 fixation rates of free-living cultures were assayed by acetylene reduction (9). For symbiosis tests, *Sesbania rostrata* seedlings were stem inoculated with test *Azorhizobium* strains; after 14 days, stem nodules were excised and assayed for acetylene reduction activity (14).

Cell envelope O_2 consumption activities. Strains were batch cultured (200 ml) in MM medium, harvested by centrifugation, and washed in 2XP buffer. Cells were lysed by ultrasonication, and unbroken cells were removed by centrifugation (10 min) at 5,000 × *g*. Cell extracts were centrifuged for 1 h at 200,000 × *g*, and the pellet was resuspended (1.0 ml) in 80 mM potassium phosphate buffer, pH 7.0. O_2 consumption was measured at 30°C with an O_2 electrode unit (Hansatech) equipped with a Clark electrode. Samples (1.0 ml) in 2XP buffer were supplemented with membrane suspension (10 to 50 μl) and one of three substrates: potassium succinate (10 mM), NADH (1.0 mM), or potassium ascorbate-TMPD (10 mM–1.0 mM; Sigma Chemical Co.). Total protein in cell membrane suspensions was assayed by the method of Bradford (4).

RESULTS

Isolation and characterization of *Azorhizobium* cytaa_3 oxidase mutants. An *Azorhizobium* genomic library was constructed in λEMBL3 (15) and then probed by plaque DNA hybridization (Materials and Methods) for recombinant phages showing homology to *Bradyrhizobium coxA*, one of three contiguous genes encoding cytaa_3 oxidase (17, 30). In these experiments, plasmid pCA1 (Table 1), which carries *coxA* internal coding sequences, served as the hybridization probe. These experiments yielded λCya1, which subsequently proved to carry the homologous *Azorhizobium coxA* locus. λCya1 insert DNA was isolated and physically mapped with several endonucleases (Fig. 1a). By Southern hybridization analysis, the *Azorhizobium coxA* locus was carried on a single *Eco*RI fragment, which spanned the entire *coxA* homologous region, and more. This 5.2-kb *Eco*RI fragment was subcloned into the *Eco*RI site of plasmid pSUP202 (Table 1) chloramphenicol acetyltransferase gene, yielding plasmid pKAR1 (Table 1; Fig. 1a).

E. coli HB101/pKAR1 was infected with λ429 Tn*5*::*uidA7* (Table 1) at high multiplicity of infection; infected bacteria were plated on LB medium containing kanamycin (50 μg ml^{-1}) at 30°C. Some 300 Km^r colonies on a single plate were pooled, and plasmid DNAs were extracted (Materials and Methods). The resulting heterologous plasmid mix was used en masse to transform *E. coli* DH5α; transformation mixes were then plated on LB agar supplemented with kanamycin (50 μg ml^{-1}), ampicillin (100 μg ml^{-1}), and tetracycline (10 μg ml^{-1}). To map pKAR1 Tn*5*::*uidA7* insertions, plasmid DNA from several different Km^r Tc^r Ap^r colonies was extracted and digested with diagnostic endonucleases. Plasmids pKARG1, pKARG2, and pKARG3 (Table 1), which all contained insertions in the cloned pKAR1 *Eco*RI fragment, were isolated (Fig. 1a). These three plasmids were conjugated from *E. coli* DH5α to *Azorhizobium* wild-type strain 57100 in a triparental mating using *E. coli* HB101/pRK2073 (Table 1) as the helper. Five independent *Azorhizobium* Km^r Tc^r transconjugates from each mating were isolated and physically mapped by Southern

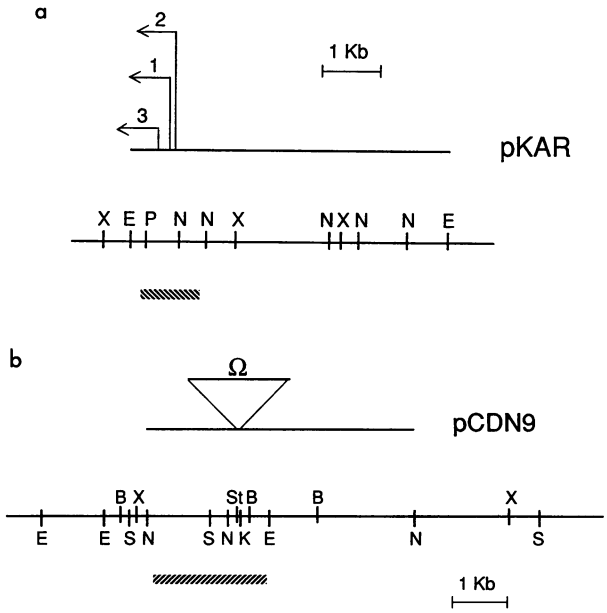

FIG. 1. Physical maps of *Azorhizobium coxA* and *cydAB* loci. Subcloned regions are denoted above each locus. Plasmid names and insertions used for reverse genetics are also identified. Endonuclease sites: B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; N, *Nco*I; P, *Pst*I; S, *Sal*I; St, *Sst*I; X, *Xho*I. (a) *Azorhizobium coxA* locus. Tn*5uidA7* (Km^r) insertions in pKAR plasmids, labeled 1, 2, and 3, reflect plasmid designations (pKARG-1, -2, and -3); arrows denote polarity of the *uidA* reading frame. The DNA region with homology to the *B. japonicum coxA* gene is indicated by the hatched bar. (b) *Azorhizobium cydAB* locus. Plasmid pCDN9SS carries a Sm^r Sp^r Ω DNA fragment inserted into pCDN9. The hatched bar denotes DNA homology with *A. vinelandii cydAB*.

hybridization and endonuclease analysis. As the parent ColE1 plasmid could not replicate in *Azorhizobium* spp., all Km^r Tc^r transconjugates proved partial *coxA* merodiploids; they carried plasmid cointegrates produced by single, homologous recombination at *coxA* (25). To obtain double recombinants carrying putative gene replacements, partial *coxA* merodiploid strains were cultured in rich (GYPCS; Materials and Methods) liquid medium without tetracycline, subcultured daily (9 days), and then plated (200 CFU per plate) on rich medium containing kanamycin. Candidates for perfect gene replacements were then screened for a Tc^s phenotype, and *coxA*::Tn*5uidA7* Tc^s Km^r haploid derivatives were obtained.

Similarly, pKARG1, pKARG2, and pKARG3 served as DNA templates for, respectively, *Azorhizobium* Tc^s Km^r candidate cytaa_3 oxidase mutants 64001, 64002, and 64003 (Table 1). From each of these three candidate strains, total genomic DNA was isolated and digested with endonuclease *Xho*I (which cuts Tn*5uidA7* once but not the *Azorhizobium coxA* coding sequence). Genomic DNA fragments were then probed by Southern hybridization with the same *Bradyrhizobium coxA* gene fragment used to identify *Azorhizobium* pKAR1 insert DNA. In strains 64001, 64002, and 64003, the *Azorhizobium* wild-type *coxA* fragment was absent and, instead, two *coxA* homologous fragments were identified. Because the wild-type, homologous DNA fragment had been split into two (Fig. 2a), all three strains were *coxA* haploids and thus carried *coxA*::Tn*5uidA7* alleles. By inference, strains 64001, 64002, and 64003 were cytaa_3 oxidase mutants and carried perfect *coxA* gene replacements. Another band of slight homology, visible at high molecular weight in all strains tested, indicated the

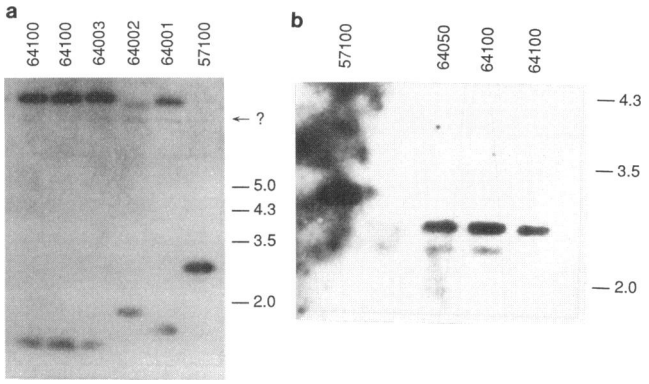

FIG. 2. Genomic Southern hybridization of *Azorhizobium* wild-type and cytochrome oxidase mutants. Lanes carry strain numbers from which genomic DNA was isolated; strain 64100 DNA was tested from two separate isolates. Bars indicate positions of relevant λ phage DNA standards (in kilobase pairs). (a) *Azorhizobium* genomic DNA cut with *Xho*I and probed with a *B. japonicum coxA* gene fragment (see text); an arrow with a question mark points to an additional band of weak *coxA* homology. (b) *Azorhizobium* genomic DNA cut with *Eco*RI and probed with an *A. vinelandii cydAB*-internal DNA fragment.

presence of a second *coxA* homologous locus which was not affected by the Tn*5uidA7* insertions.

Isolation and characterization of *Azorhizobium* cyt*d* oxidase mutants. The *Azorhizobium* genomic library in λEMBL3 was similarly probed by DNA hybridization with plasmid pMH3, which carried the subcloned *A. vinelandii cydAB* locus encoding cyt*d* oxidase (Table 1) (26); three cross-hybridizing recombinant phages, λCyd1, λCyd2, and λCyd3, were identified. From subsequent physical mapping, all three phages carried insert *Azorhizobium* DNA derived from the same locus (Fig. 1b). From Southern hybridization experiments, all *cydAB* homologous *Azorhizobium* genomic sequences comprised two adjacent endonuclease *Nco*I fragments. This 5.0-kbp DNA fragment presumably carried the *cydAB* genes. To subclone this locus, phage λCyd1 was partially digested with endonuclease *Nco*I; the 5.0-kbp DNA fragment was isolated and inserted into the pSUP202 chloramphenicol acetyltransferase gene, yielding plasmid pCDN9 (Table 1; Fig. 1b).

Plasmid pCDN9 was physically mapped, and unique *Sac*I and *Kpn*I sites within the inferred *Azorhizobium cydAB* coding region were identified (Fig. 1b). These *Sac*I and *Kpn*I sites were targeted for insertion mutagenesis by the λ DNA fragment, which carries a streptomycin-spectinomycin phosphotransferase gene (Table 1). To generate a λ DNA fragment cassette, plasmid pCAP66 (Table 1) was digested with *Hin*dIII, and the λ DNA fragment was cloned into pBluescriptSK+ at the *Hin*dIII site. The resulting plasmid was then digested with both *Sac*I and *Kpn*I, and the λ DNA fragment was reisolated and then ligated into *Sac*I and *Kpn*I double-digested pCDN9. From physical mapping and DNA hybridization experiments, the resulting Sm^r Sp^r plasmid, pCDN9SS, carried *Azorhizobium* DNA in which the genomic, *cydAB*-internal fragment had been replaced by the λ DNA fragment (data not presented).

As described above for *coxA* mutants, plasmid pCDN9SS was then used to construct *Azorhizobium* cyt*d* oxidase mutants carrying perfect gene replacements. To assess Sm^r Sp^r candidate strains for *cydAB* gene replacement, endonuclease *Eco*RI-digested genomic DNA from Sm^r Sp^r Tc^s derivative 64050 was probed with an *Azotobacter cydAB*-internal pMH3 DNA fragment (Fig. 2b). Whereas *Eco*RI did not cut *Azorhizobium cyd* sequences, it did cut the λ DNA fragment once. Indeed, the wild-type *cydAB* fragment was absent from strain 64050 and was replaced by two smaller *cydAB* homologous fragments. Strain 64050 had thus lost some 400 bp of *cyd* DNA (the *Sac*I-*Kpn*I DNA fragment) and had gained the λ DNA fragment at this locus. Strain 64050 thus carried a *cydAB*::Ω allele. By inference, strain 64050 was a cyt*d* oxidase mutant and carried a perfect gene replacement (Table 1).

Construction of an *Azorhizobium* cytaa_3 oxidase, cyt*d* oxidase double mutant. *E. coli* DH5α/pDCN9SS was used as the conjugal donor in triparental matings with *E. coli* HB101/pRK2073 as the helper and *Azorhizobium coxA* mutant 64003 as the recipient. *Azorhizobium* transconjugants were identified as Km^r Sm^r Sp^r Tc^r colonies (Materials and Methods). By physical mapping (data not presented), these multiply resistant 64003 derivatives proved *cydAB* merodiploids (genome:: DCN9SS cointegrates). As described above, strain 64003 derivatives were repeatedly subcultured without antibiotic selection, subcultures were plated for Km^r colonies, and colonies were then screened for a Tc^s phenotype. By genomic Southern hybridization analysis, one such isolate, strain 64100, proved haploid for both *cydAB* and *coxA* loci. Indeed, strain 64100 carried both *cydAB*::Ω and *coxA*::Tn*5uidA7* alleles (Fig. 2). By inference, strain 64100 was a cytaa_3 oxidase, cyt*d* oxidase double mutant and carried perfect gene replacements at these loci (Table 1).

Isolation and characterization of *Azorhizobium* pleiotropic cyt*c* mutants. We previously isolated *Azorhizobium* mutants unable to catabolize nicotinate. Among these mutants, we identified a pleiotropic phenotype subclass (24). Not only were these pleiotropic mutants unable to hydroxylate nicotinate ($Nic[N]^-$ phenotype), they were also unable to oxidize TMPD ($TMPD^-$ phenotype) (24). These mutants were also unable to utilize glutarate (Glr^- phenotype) as a C source (23). Therefore, a random VP2021 insertion library of the *Azorhizobium* wild type (Materials and Methods) was screened for mutants unable to oxidize TMPD. Three independent $TMPD^-$ mutants were obtained; all proved $Nic[N]^-$, $TMPD^-$, and Glr^- and precisely mirrored the previously isolated $Nic[N]^-$ mutants.

Mutants isolated from both screens shared other phenotypes. When allowed to grow for more than 3 days in liquid minimal salts medium, all strains excreted pink compounds that strongly fluoresced. From both UV-visible absorbance and fluorescence emission spectra, the predominant substance proved identical to coproporphyrin IX (Fig. 3). As with *Rhodobacter capsulatus* (6), from whole-cell visible spectrophotometry, these mutants lacked all cyt*c* chromophores ($cytc^-$ phenotype; Fig. 4). These pleiotropic cyt*c* mutants might be affected in the maturation of mature cyt*c* and/or be unable to cysteinylate heme with *c*-type apocytochromes. Because all mutants characterized here were phenotypically indistinguishable, we chose $TMPD^-$ strain 61207 (Table 1) as representative for more detailed analyses, discussed below.

Reduced-minus-oxidized visible light spectrophotometry of cytochrome oxidase mutants confirms genetic phenotypes and allows diagnostic peak assignments. Reduced-minus-oxidized visible light difference absorbance spectra were taken (Materials and Methods) from whole cells grown to early exponential phase in MM medium (Fig. 4). All strains showed *b*-type cytochrome absorbance maxima (peaks) at approximately 535 and 560 nm. Among strains analyzed, the spectral region from 580 to 650 nm varied most. Wild-type strain 57100 showed a broad absorbance peak at 605 nm and a second, discrete peak at 630 nm. By contrast, cytaa_3 mutant 64003 showed a shoulder and cyt*c* mutant 61207 showed a peak at 595 nm, presumably

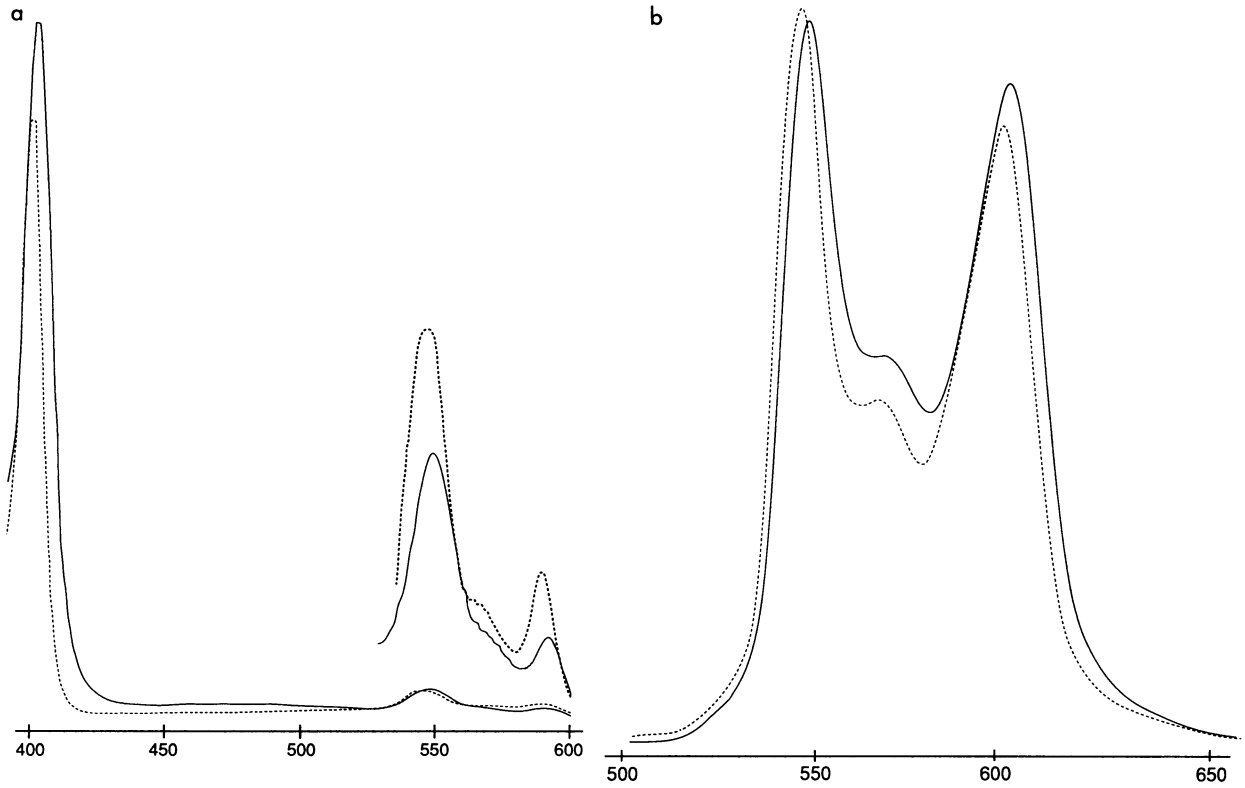

FIG. 3. Visible absorbance and fluorescence emission spectra of an excreted substance purified from strain 61207 cultures compared with an authentic sample of coproporphyrin IX (Sigma) in aqueous solution, pH 1.0. (a) Visible absorbance spectrum of coproporphyrin IX (dotted line) and sample (solid line). (b) Fluorescence emission spectrum with excitation beam at 400 nm of coproporphyrin IX (dotted line) and sample (solid line).

due to a cytb_{595} component of a cyt*bd* oxidase complex, and both showed a cyt*d* oxidase peak at 630 nm (1, 18, 21, 31). In both cytaa_3 mutant 64003 and cyt*c* mutant 61207, this finding implied increased cyt*d* oxidase relative to the wild type. The cyt*d* mutant 64050 lacked both 595- and 630-nm peaks but did show a peak at 605 nm, presumably due to cytaa_3 oxidase (1, 29, 30, 31).

cytaa_3, cyt*d* double mutant 64100 showed a new peak at 610 nm, which might represent an alternative *a*-type cytochrome oxidase. From line shape analyses (data not presented), the broad peak at 605 nm observed in wild-type 57100 might well represent a composite of the 605-nm (cytaa_3) peak and the 610-nm (alternative *a*-type cytochrome) peak. This 610-nm peak was not observed in the spectrum of cyt*d* oxidase mutant 64050. By analogy to the genus *Bradyrhizobium*, its closest phylogenetic relative, the absorbance peak at 610 nm might be diagnostic for the alternative *a*-type heme/copper oxidase whose genes have been cloned and sequenced (8). Because strain 61207 showed a trough at 605 to 610 nm, all *a*-type cytochrome activities were absent in this pleiotropic cyt*c* mutant. Therefore, the second *a*-type cytochrome might also be a cyt*c* oxidase.

In all cases, spectrophotometric data were consistent with DNA mapping data and therefore served to confirm the phenotypes of cytochrome mutants constructed by DNA insertion and gene replacement. Not surprisingly, partial DNA sequencing of pKAR1 in its *coxA* homologous region showed a very high degree of homology with *Bradyrhizobium coxA*, the source of the hybridization probe used to isolate the *Azorhizobium coxA* locus (data not presented).

In CO-bound-reduced-minus-reduced spectra, *Azorhizobium* membranes show both cyt*o* and cytc_{550} oxidase activities. CO-bound-reduced-minus-reduced visible light absorbance spectra were taken from cell envelope preparations (Materials and Methods) of representive cytochrome mutants grown to early exponential phase (Fig. 5a and b). All strains showed a 560-nm trough and 530- and 570-nm peaks diagnostic for *o*-type cytochromes (1, 31). In addition, wild-type strain 57100, cytaa_3 mutant 64003, and cyt*c* mutant 61207 all showed a 645-nm peak, characteristic of a *d*-type cytochrome (1, 31). Consistent with their cytochrome mutant phenotypes, cyt*d* mutant 64050 and cytaa_3, cyt*d* mutant 64100 both lacked this 645-nm peak. Both strains 57100 and 64050 showed 590-nm peaks diagnostic for cytaa_3 oxidase. However, both cytaa_3 mutant strain 64003 and double mutant 64100 showed a persistent 590-nm shoulder, consistent with the presence of a second *a*-type cytochrome oxidase.

CO-bound-reduced-minus-reduced spectra were also taken from late exponential-phase cultures (Materials and Methods). Interestingly, cytaa_3 mutant 64003 and cytaa_3, cyt*d* double mutant 64100 both showed an additional 550-nm trough indicative of a CO-binding, *c*-type cytochrome (Fig. 5c). Indeed, cyt*c* mutant strain 61207 did not show this 550-nm trough (data not presented). The CO-binding cytc_{550} signal was not observed in membranes isolated from early exponential-phase cells (Fig. 5b). As also observed in the reduced-minus-oxidized difference spectra, early exponential-phase membranes had much less cyt*c* activity. As evidenced by decreases in the 590-nm shoulder for both wild-type 57100 and cyt*d* mutant 64050, cytaa_3 activities in these late exponential-phase cultures

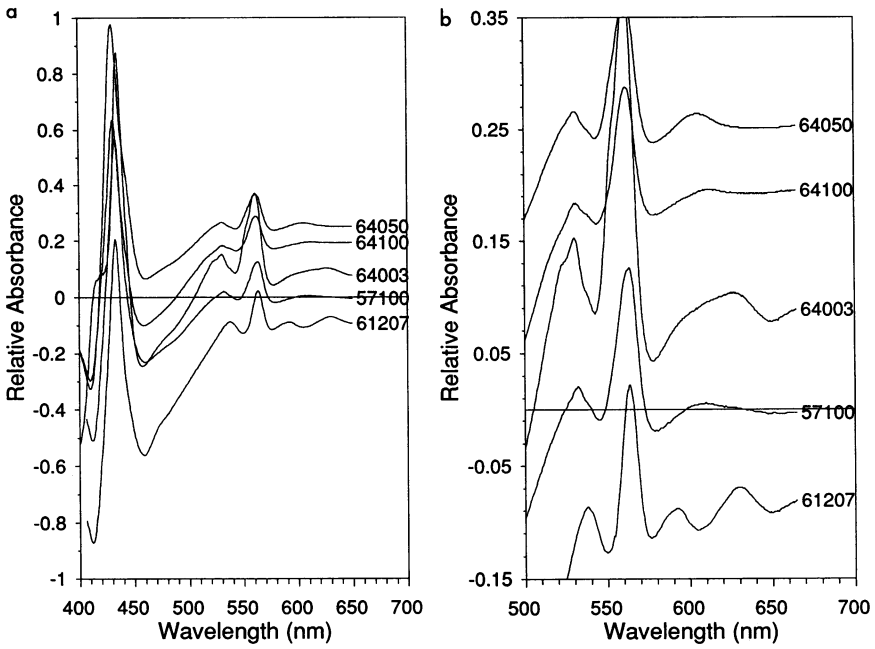

FIG. 4. Reduced-minus-oxidized difference absorbance spectra of dodecylmaltoside-solubilized membranes isolated from *Azorhizobium* cytochrome mutants grown to early exponential phase in GYPCS medium. (a) The entire 400- to 500-nm spectral region. (b) Closeup of the 500- to 700-nm spectral region, which details differences among the strains studied. All spectra were taken at 0.5-nm intervals and are presented as raw data.

were much lower. Upon entry to stationary phase, cytc_{550} oxidase might be induced, and cytaa_3 oxidase might be inhibited, in response to low-O_2 stress brought about by high batch culture densities.

***Azorhizobium* cytaa_3 oxidase genes are active in aerobic batch cultures during exponential phase but are inactive at the onset of stationary phase.** At target genes, Tn*5uidA7* insertions yield β-glucuronidase transcriptional fusions (36). Indeed, for strains 64001, 64002, and 64003 (all carrying *coxA*::Tn*5uidA7* haploid alleles), colonies fluoresced strongly when tested with

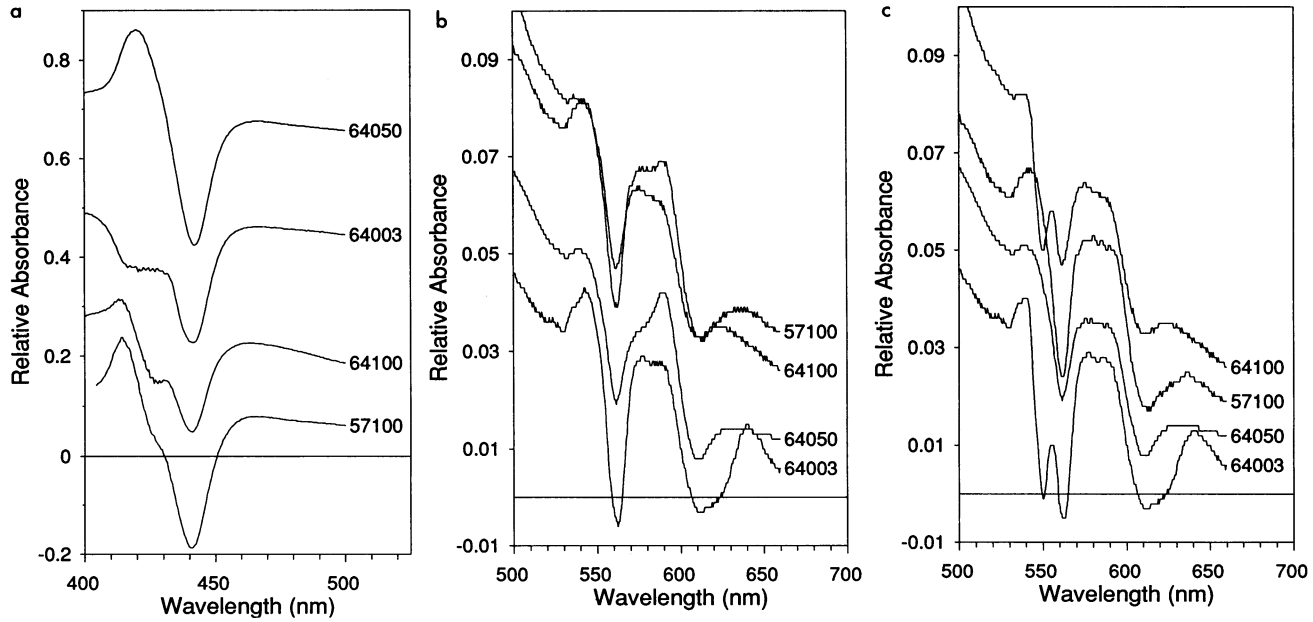

FIG. 5. CO-bound reduced-minus-reduced difference absorbance spectra of dodecylmaltoside-solubilized membranes isolated from *Azorhizobium* cytochrome oxidase mutants. (a and b) The 400- to 500-nm (a) and 500- to 700-nm (b) spectral regions of cultures grown to early exponential phase in GYPCS medium. (c) The 500- to 700-nm spectral region of cultures grown to late exponential phase in GYPCS medium. All spectra were taken at 0.5-nm intervals and are presented as raw data.

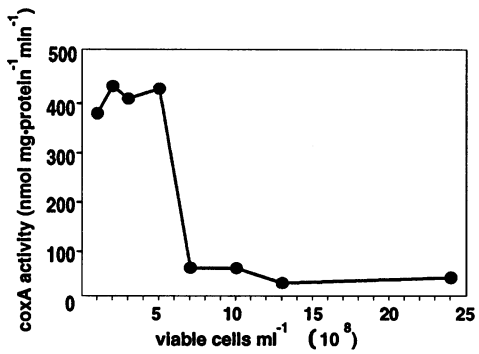

FIG. 6. *Azorhizobium coxA* gene expression versus culture status. Genomic *coxA*::*uidA* fusion allele activity was measured as β-glucuronidase activity (nanomoles of 4-methylumbelliferyl-β-D-glucuronide hydrolyzed per minute per milligram of protein; mean value of two parallel experiments).

the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide. Thus, the Tn*5uidA7* insertions carried by strains 64001, 64002, and 64003, themselves in the same genomic orientation, all paralleled the *coxA* reading frame (data not presented). As discussed above, difference spectra taken from cells in late exponential or stationary culture phase (Materials and Methods) showed very low cytaa_3 activity. Therefore, cytaa_3 mutant 64003 liquid batch culture samples were assayed for *coxA* gene activity via the β-glucuronidase reporter at various times (Fig. 6). High levels of β-glucuronidase activity, indicative of high levels of *coxA* gene expression, were obtained and were maintained until cultures reached late exponential phase. Relative β-glucuronidase activity then dropped by a factor of 7 and remained low in stationary phase. Therefore, cytaa_3 oxidase genes were strongly expressed during exponential growth phase and then deactivated before the onset of stationary phase.

Growth rates of *Azorhizobium* cytaa_3 mutants, cyt*d* mutants, and cytaa_3, cyt*d* double mutants are essentially wild type. Cytochrome mutant growth rates were measured in batch cultures with one rich medium and two defined media, which contained either ammonium or nicotinate as the N source (data not presented). Inoculant cultures were diluted to 1×10^7 cells ml^{-1} and then vigorously shaken at 30°C. Culture samples were removed hourly, and growth was estimated by increase in 600-nm light scattering, which had been previously calibrated against viable cell counts. Cultures grew exponentially until cell density reached at least 10^9 cells ml^{-1}. Surprisingly, all cytochrome mutants showed cell doubling times similar to that of the wild type. Interestingly, pleiotropic cyt*c* mutant 61207 proved exceptional; it did not grow on nicotinate as the N source, and it grew at one-third of the wild-type rate in rich medium (data not presented). This phenotype was also visible on solid media; representative cyt*c* mutant 61207 and all TMPD$^-$ mutants yielded small colonies on rich medium but wild-type colonies on minimal medium.

Whereas cytaa_3 mutants fix N_2 at wild-type rates, cyt*d* mutants are partially defective, and pleiotropic cyt*c* mutants are completely defective. All cytochrome oxidase mutants grew on N_2 as the sole N source and effectively nodulated the host plant *S. rostrata*. The cytaa_3 mutant 64003 was fully wild type for both symbiotic and free-living N_2-fixing ability (Table 2). In optimized N_2-fixing cultures (9), both cyt*d* mutant 64050 and cytaa_3 oxidase, cyt*d* oxidase double mutant 64100 showed a 40% decrease in N_2 fixation rates (Table 2). This decrease was also reflected in decreased symbiotic N_2 fixation rates by *S. rostrata* stem nodules elicited with these strains. Of the two, the double mutant 64100 showed lower symbiotic N_2 fixation rates. By contrast, the pleiotropic cyt*c* mutant 61207 showed <10% of wild-type N_2 fixation rates both in culture and in planta (Table 2).

TABLE 2. Dinitrogenase activities of *Azorhizobium* cytochrome oxidase mutants

Strain	Phenotype	N_2 fixation status	
		3% O_2 culture[a] (nmol of ethylene h^{-1} 10^8 cells^{-1})	*S. rostrata* nodules (nmol of acetylene-dependent ethylene h^{-1} g of fresh nodule wt^{-1})
57100	Wild type	2.6	25.0
61207	cyt*c*$^-$	0.1	<1.0[b]
64003	cyt$aa_3$$^-$	2.9	25.0
64050	cyt*d*$^-$	1.6	15.0
64100	cyt$aa_3$$^-$ cyt*d*$^-$	1.8	7.3

[a] Mean value of three parallel experiments; standard error did not exceed 15%.
[b] Tiny nodules; no detectable leghemoglobin.

Cell envelope O_2 consumption by cytochrome oxidase mutants. *Azorhizobium* cell envelopes were prepared (Materials and Methods) from each mutant and then tested for oxidase activity with several e$^-$ donors (Table 3). Again, pleiotropic cyt*c* mutant 61207 was most affected, showing 50, 30, or <10% of wild-type activity with either NADH, succinate, or ascorbate-TMPD, respectively, as the e$^-$ donor. The cytaa_3 mutant 64003, cyt*d* mutant 64050, and cytaa_3, cyt*d* double mutant 64100 showed no loss of NADH-dependent oxidase activity. All mutants showed <50% activity with succinate as the e$^-$ donor. As expected, cytaa_3 mutants 64003 and 64100 both showed reduced oxidase activity with ascorbate-TMPD as the e$^-$ donor. However, this activity loss was not apparent when colonies were examined for in vivo production of the blue color indicative of TMPD oxidation (Materials and Methods). The cyt*d* mutant 64050 showed essentially wild-type oxidase activity with ascorbate-TMPD as the e$^-$ donor (Table 3).

DISCUSSION

For oxidative phosphorylation, cyt*c*-dependent respiration is most efficient and, a priori, best suited for the high energy demands of N_2 fixation (1, 31, 40). Unlike those of quinol oxidases, cyt*c* respiratory chains employ an additional, cytbc_1 complex which augments proton pumping. In *Bradyrhizobium* spp., cytbc_1 is required for symbiotic N_2 fixation; *fbcF* and *fbcH*

TABLE 3. Terminal oxidase activities measured by O_2 consumption rates

Strain	Phenotype	Activity[a] (nmol of O_2 min^{-1} mg of protein^{-1})		
		Ascorbate-TMPD[b]	Succinate	NADH
57100	Wild type	87	127	151
61207	cyt*c*$^-$	6	43	70
64003	cyt$aa_3$$^-$	12	30	149
64050	cyt*d*$^-$	51	29	162
64100	cyt$aa_3$$^-$ cyt*d*$^-$	13	39	151

[a] Mean value of at least two experiments; standard error did not exceed 15%.
[b] e$^-$ donor.

mutants, which lack the cytbc_1 complex but retain other *c*-type cytochromes, yield Fix$^-$ nodules on soybean (41). In the present study, *Azorhizobium* strains lacking all cyt*c* activity were impaired in both free-living and symbiotic N_2 fixation. However, because the cyt*c* mutants isolated and analyzed here were pleiotropic, the importance of *Azorhizobium* cytbc_1-dependent respiration for N_2 fixation was not unambiguous. Other attributes, such as generally lower oxidase activities, might contribute to observed phenotypes.

Bradyzhizobium coxA mutants exhibit both slow growth in rich medium and a TMPD$^-$ phenotype, and they fix N_2 at wild-type rates in symbiosis (27, 29). The same is true for *Rhizobium leguminosarum* bv. phaseolus mutants which lack cytaa_3 but retain *c*-type cytochromes (27). However, cytaa_3 may play a larger role in early nodulation events, such as during rapid bacterial proliferation. For example, *R. leguminosarum* bv. phaseolus mutants which overexpress cytaa_3 nodulate host beans more quickly, and fix N_2 earlier than does the wild type (39). A role for cytaa_3 in *Azorhizobium* symbiosis has not been demonstrated. As reported here, the *Azorhizobium coxA* gene was most highly expressed when cells grew rapidly in O_2-rich, nutrient-rich environments. Indeed, in *Azorhizobium* chemostat cultures, cytaa_3 activity also decreased as dissolved O_2 tension decreased (40). Consistent with the regulation of *coxA* gene expression, little cytaa_3 activity was evident in spectra from late exponential-phase batch cultures.

In early exponential phase, cytaa_3 mutants still showed *a*-type cytochrome activity. Both cytaa_3 mutant 64003 and cytaa_3, cyt*d* mutant 64100 showed normal growth rates when batch cultured in both rich and defined media, and fixed N_2 at essentially wild-type rates. Therefore, phenotypic compensation by an alternative cyt*c* oxidase is implicit. Because pleiotropic cyt*c* mutant 61207 is devoid of all *a*-type cytochrome activities, all *a*-type cytochromes are apt to be cyt*c* oxidases. Evidence for a second *a*-type cytochrome oxidase was also supported by CO difference spectra, in which the cytaa_3 mutants continued to show a 590-nm shoulder diagnostic for *a*-type cytochrome oxidase activity. In its reduced-minus-oxidized difference spectrum, cytaa_3, cyt*d* double-mutant strain 64100 showed a peak at 610 nm, not the 605-nm peak present in the cyt*d* mutant strain 64050. Whether the second *a*-type cytochrome induced in cytaa_3 mutants 64003 and 64100 is a cyt*c* oxidase per se remains to be addressed. Two linked *B. japonicum* genes encoding a second *a*-type cytochrome oxidase have been identified and sequenced. In the inferred amino acid sequence for its *a*-type oxidase subunit, the conserved histidine residues which serve as heme axial ligands and are diagnostic for heme/copper oxidases are indeed present. However, its upstream, presumed immediate e^- donor subunit sequence shows no homology with that of other purple bacterial cytaa_3 oxidases (8). As the e^- donor subunit might well interact with cyt*c*, there is then some reason to suppose that the second *a*-type cytochrome oxidase might not be cyt*c* dependent. However, because *Bradyrhizobium* spp. and other purple bacteria possess numerous cyt*c* species (34), heterologous e^- donor subunits for *a*-type cytochromes might reflect distinct cyt*c* specificities.

In genomic hybridization experiments with *Bradyrhizobium coxA* as the probe, under lowered stringency, a second, weakly hybridizing *Azorhizobium* gene homolog was evident (Fig. 2a). This weak signal may result from the gene encoding the cyt*bo* oxidase heme/copper oxidase subunit (11). More likely, this hybridization signal is due to the second, *a*-type cytochrome oxidase genes. Indeed, both the cytaa_3 oxidase genes and those of its *a*-type homolog were isolated from *B. japonicum* by using a *Paracoccus coxA* probe (7, 8).

In CO difference spectra taken from late exponential-phase cultures, both cytaa_3 mutant 64003 and cytaa_3, cyt*d* double mutant 64100 showed a cytc_{550} CO-binding chromophore not detectable in the wild type. A similar cytc_{550} peak was visible in CO-bound-reduced-minus-reduced difference spectra of *Rhizobium* sp. bv. viciae bacteroids (2). In addition, two CO-binding chromophores, a membrane bound cytc_{552} and a soluble cytc_{555}, are present in *Bradyrhizobium* bacteroids (2). Because mutations in either the *Bradyrhizobium* cytc_{552} or cytc_{555} gene do not affect symbiotic N_2 fixation, the function of these cytochromes remains obscure (34, 42, 43). The observed *Azorhizobium* cytc_{550} activity might, then, essentially be that of a bacteroid cytochrome, which is expressed in cytaa_3 mutants 64003 and 64100 late in batch culture cycle (when these cells are under low O_2 stress due to high culture density) to compensate for loss of wild-type oxidase activities.

Azorhizobium cyt*o* oxidase, not studied here, might yet play important physiological roles in growth and in symbiotic N_2 fixation. Indeed, cyt*o* signals are present in the spectra of bacteroids taken from several *Rhizobium* spp. (3, 40). Because pleiotropic *Azorhizobium* cyt*c* mutants used both cyt*o* and cyt*d* as terminal oxidases and grew normally in defined media, cyt*o* appears to be a quinol oxidase, as is true for enteric bacterial cyt*o*. Conceivably, cyt*o* might have both quinol and cyt*c* oxidase activities. In heterologous *E. coli* derivatives carrying the complete set of *Klebsiella* N_2 fixation genes, cyt*d* oxidase mutants are N_2 fixation defective, and thus *E. coli* cyt*o* oxidase is superfluous (19).

For *Klebsiella* and *Azotobacter* spp., which are free-living diazotrophs, cyt*d* oxidase mutants are completely unable to fix N_2 in either aerobic or microaerobic culture (19, 22, 26, 38). Although *Azorhizobium* cyt*d* oxidase mutant strains 64050 and 64100 were still able to fix appreciable N_2 both in culture and in planta, N_2 fixation rates were significantly decreased. While *Azotobacter* cyt*d* oxidase mutants cannot fix N_2 under 21% O_2, they grow quite well under 1.5% O_2 (22). In *Azorhizobium* N_2-fixing cultures, cyt*d* oxidase activity is not similarly critical. Rather, cyt*d* oxidase must function in concert with one or more additional terminal oxidases.

In summary, no single terminal oxidase seems essential for any particular *Azorhizobium* growth regimen. Quite the contrary, under all physiological conditions studied, azorhizobia simultaneously use multiple terminal oxidases. Indeed, azorhizobia readily reformulate their mix of terminal oxidases. In aerobiosis with defined media, all cytochrome oxidase mutants grew normally. The pleiotropic cyt*c* mutant 61207 increased its cyt*d* content, possibly to compensate for its loss of *c*-type cytochromes. The cytaa_3 mutants 64003 and 64100 expressed an alternative *a*-type oxidase, and strain 64003 also showed increased cyt*d* oxidase levels. Indeed, among the rhizobia, the unique ability of *Azorhizobium* spp. to fix N_2 in near aerobiosis may be due both to a cyt*d* terminal oxidase and to the flexibility to alter its terminal oxidase composition in response to changes in O_2 environments. Indeed, this flexibility might also prove adaptive for symbiotic N_2 fixation.

During early legume nodule development, invading rhizobia grow rapidly and proliferate extensively. As they migrate to the interior of the developing nodule cortex, and as nodule bacterial counts mount exponentially, rhizobia might experience very different O_2 environments (3). Therefore, during proliferation and prior to both leghemoglobin induction and bacteroid differentiation, infecting rhizobia might need to modulate both the O_2 affinity of respiratory terminal oxidases and the efficiency with which ATP is synthesized. Identification of the *Azorhizobium* terminal oxidase(s) primarily responsible for respiration in the O_2-deficient environment of the mature

nodule would then be particularly problematic. Any terminal oxidase mutation which slows, or halts, bacterial proliferation during nodule development would produce a nodule with few bacteroids, resulting in a lowered rate of symbiotic N_2 fixation. Thus, the low symbiotic N_2 fixation rates observed with *Azorhizobium* cyt*d* mutant 64050 and cytaa_3, cyt*d* double mutant 64100 might reflect poor bacterial proliferation. Indeed, while 64100 exhibited symbiotic N_2 fixation rates even lower than those of cyt*d* mutant 64050, it fixed N_2 in culture similarly. Quite possibly, all pleiotropic cyt*c* mutant or cyt*c* oxidase mutant rhizobia, comprising several genera and species (27–29, 41), might proliferate poorly during nodule development.

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