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A Cytochrome cbb_3 (Cytochrome *c*) Terminal Oxidase in *Azospirillum brasilense* Sp7 Supports Microaerobic Growth

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Spectral analysis indicated the presence of a cytochrome cbb_3 oxidase under microaerobic conditions in *Azospirillum brasilense* Sp7 cells. The corresponding genes (*cytNOQP*) were isolated by using PCR. These genes are organized in an operon, preceded by a putative anaerobox. The phenotype of an *A. brasilense cytN* mutant was analyzed. Under aerobic conditions, the specific growth rate during exponential phase (μ_e) of the *A. brasilense cytN* mutant was comparable to the wild-type specific growth rate (μ_e of approximately 0.2 h^{-1}). In microaerobic NH_4^+-supplemented conditions, the low respiration of the *A. brasilense cytN* mutant affected its specific growth rate (μ_e of approximately 0.02 h^{-1}) compared to the wild-type specific growth rate (μ_e of approximately 0.2 h^{-1}). Under nitrogen-fixing conditions, both the growth rates and respiration of the wild type were significantly diminished in comparison to those under NH_4^+-supplemented conditions. Differences in growth rates and respiration between the wild type and the *A. brasilense cytN* mutant were less pronounced under these nitrogen-fixing conditions (μ_e of approximately 0.03 h^{-1} for the wild type and 0.02 h^{-1} for the *A. brasilense cytN* mutant). The nitrogen-fixing capacity of the *A. brasilense cytN* mutant was still approximately 80% of that determined for the wild-type strain. This leads to the conclusion that the *A. brasilense* cytochrome cbb_3 oxidase is required under microaerobic conditions, when a high respiration rate is needed, but that under nitrogen-fixing conditions the respiration rate does not seem to be a growth-limiting factor.

Azospirillum brasilense is a gram-negative soil bacterium that lives in the rhizospheres of various plants, such as maize, wheat, and rice. When combined nitrogen is available, this bacterium is able to grow in anaerobic, microaerobic, or aerobic conditions. Under anaerobic conditions, when NO_3^- is available, denitrification provides the energy for growth (28, 29). Under microaerobic conditions, *A. brasilense* can reduce molecular N_2 in the absence of combined nitrogen. In aerobic or microaerobic conditions, O_2 is used as terminal electron acceptor (17). Like many other bacteria, *A. brasilense* has a branched respiratory chain. The presence of a respiratory chain that efficiently couples electron transfer with proton pumping at low oxygen concentrations is inferred from the attraction of *A. brasilense* to low oxygen concentrations. Under these conditions, a maximal proton motive force is generated (3, 53). The existence of a high-affinity terminal oxidase and a second oxidase with a significantly lower affinity in *A. brasilense* Sp7 was previously noted (4). Moreover, depending on the O_2 status of the culture, *A. brasilense* Sp7 and Cd showed marked differences in cytochrome content (6, 21, 31, 34). For both strains spectral analysis revealed evidence for the presence of cytochrome *b* (α peak at 560 nm in the reduced-minus-oxidized difference spectrum), cytochrome *c* (α peak at 552 nm in the reduced-minus-oxidized difference spectrum), and a CO-binding *o*-like cytochrome (α peak at 558 nm in the reduced-minus-oxidized difference spectrum and a trough at 560 nm in the CO-reduced-minus-reduced difference spectrum) (6, 21, 34). The amounts of cytochromes *b* and *c* increased as the O_2 concentration was lowered (6, 21, 31, 34). In contrast to the case for *A. brasilense* Sp7, a cytochrome *d* (peak at 628 nm in the reduced-minus-oxidized difference spectrum) was found in *A. brasilense* Cd (34). A cytochrome *a* (α peak at 603 to 605 nm in the reduced-minus-oxidized difference spectrum), observed under high aeration, was present in *A. brasilense* Cd (31, 34), but in *A. brasilense* Sp7 spectral evidence for this oxidase seemed to be less clear and even contradictory (6, 21).

The cytochrome cbb_3 cytochrome *c* oxidase, encoded by the *fixNOQP* operon in rhizobial species (18, 23, 32, 38, 50) or by a similar *cco*(*cyt*)*NOQP* operon in other bacteria (7, 39, 43, 45), appears to be a cytochrome *c* terminal oxidase belonging to the heme-copper oxidase superfamily (14). In most rhizobial species this oxidase is essential for nitrogen-fixing endosymbiosis (18, 32, 50) and is characterized by an extremely high O_2 affinity (16, 33). In the bacteria *Magnetospirillum magnetoaceticum* and *Agrobacterium tumefaciens*, and in *Azorhizobium caulinodans* growing nonsymbiotically, the cbb_3-type cytochrome *c* terminal oxidase seems to be at least partially responsible for the microaerobic respiration (23, 39, 43). In *Rhodobacter capsulatus*, however, this oxidase drives aerobic respiration and does not function as the obligate oxidase during microaerobic nitrogen fixation (45). Proton pumping activity of the cytochrome cbb_3 oxidase was demonstrated in *Paracoccus denitrificans* (7).

The purpose of this study was the characterization of the terminal oxidase active during microaerobic growth in *A. brasilense*. In particular, we were interested in assessing the role of this oxidase during nitrogen fixation.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains used and plasmids described in this work are listed in Table 1. *Escherichia coli* strains

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Properties	Reference or source
E. coli		
DH5α	*hsdR17 endA1 thi-1 gyrA96 relA1 recA1 supE44* Δ*lacU169* (ϕ80*lacZ* ΔM15)	Gibco-BRL
S17-1	*thi endA recA hsdR* with RP4-2-Tc::Mu-Km::Tn*7* integrated in chromosome	41
A. brasilense		
Sp7	Wild type; ATCC 29145	44
FAJ851	*cytN* mutant; Km^r (plus direction)	This work
FAJ852	*cytN* mutant; Km^r (minus direction)	This work
Plasmids		
pEMBL8	Cloning vector; Ap^r	8
pFAJ853	pLAFR1 clone from genome bank of *A. brasilense* Sp7, containing *cytNOQP*; Tc^r	This work
pFAJ856	pSUP202 with the *Eco*RI/*Xba*I fragment from pFAJ863 blunt inserted in the *Pst*I site (minus direction); Tc^r Km^r Ap^r	This work
pFAJ857	pSUP202 with the *Eco*RI/*Xba*I fragment from pFAJ862 blunt inserted in the *Pst*I site (plus direction); Tc^r Km^r Ap^r	This work
pFAJ860	pUC18 with a 6-kb *Kpn*I fragment of pFAJ853; Ap^r	This work
pFAJ861	pUC18 with a 1.8-kb *Bam*HI fragment of pFAJ853; Ap^r	This work
pFAJ862	pFAJ861 with the Km^r cassette from pHP45Ω-Km blunt ligated in the *Apa*I site (plus direction); Ap^r Km^r	This work
pFAJ863	pFAJ861 with the Km^r cassette from pHP45Ω-Km blunt ligated in the *Apa*I site (minus direction); Ap^r Km^r	This work
pHP45Ω-Km	Ap^r Km^r	10
pLAFR1	IncP broad-host-range cosmid; Tc^r	13
pSUP202	Mobilizable plasmid, suicide vector for *A. brasilense*; Cm^r Tc^r Ap^r	41
pUC18	Cloning vector; Ap^r	51

were grown in Luria-Bertani medium at 37°C. To grow *Azospirillum*, minimal medium (MMAB) was used (49). The nitrogen-free medium used for nitrogen fixation was the MMAB medium, devoid of NH_4Cl. Solid medium contained 15 g of agar per liter. For conjugation YEP medium (containing 10 g of Bacto Peptone, 5 g of NaCl, and 10 g of yeast extract per liter) was used, and transconjugants of *Azospirillum* were selected on MMAB medium. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml; and tetracycline, 10 μg/ml.

A. brasilense was grown in a chemostat of 1.8-liter capacity (Applitek). The parameters of fermentation (pH, temperature, dissolved oxygen [DO], and air flow) were controlled by the ML-4100 fermentor control system (New Brunswick). All data from the ML-4100 system were transmitted into a computer loaded with the ASF 2.0 software (New Brunswick). DO levels were monitored with an autoclavable O_2 electrode (Ingold). During aerobic growth, the airflow rate was set at 1.8 liters/min. According to the optimal values indicated in the literature (30, 46, 48), a DO concentration of 2.5 μM (2.5 μM DO at 30°C in sterile medium = a pO_2 of 0.006 atm) was used for growth under nitrogen-fixing conditions. In order to maintain the DO at a constant level of 2.5 μM (microaerobic growth), the fermentor was sparged with a gas mixture of N_2 and air. The N_2 flow rate was set to 1.27 liters/min. The airflow rate was controlled by the ML-4100 system through a mass flow controller and automatically adapted according to the DO concentration values. The culture was stirred at a constant rate of 400 rpm. The growth temperature during fermentation was 30°C. The pH was maintained at 6.8 and adjusted with an H_3PO_4 (1 M) solution during fermentation according to the pH values measured by a pH probe (Ingold). A preculture of 100 ml, used to inoculate the fermentor, was grown in a flask of 250 ml of MMAB with NH_4^+ at 200 rpm and 30°C until an optical density at 578 nm (OD_{578}) of 1.5 was reached. If cells were intended for growth in nitrogen-fixing conditions, the preculture was washed to remove residual NH_4^+. Samples of 5 to 10 ml, used for analyzing turbidity, protein concentration in the cells, and residual malate and NH_4^+ in the supernatant, were withdrawn automatically by a Biosampler (New Brunswick) during fermentation. Samples for acetylene reduction activity were taken anaerobically at the late exponential phase and transferred into gas-tight flasks which had been flushed with the headspace gas of the fermentor to adjust the DO concentration and with 10% (vol/vol) of C_2H_2 added. After an initial incubation of 30 min at 30°C and 200 rpm, the amount of ethylene produced was measured as previously described (48). Values for the specific nitrogenase activity presented in Results are the averages from at least three independent samples, each assayed at least five times. Data were analyzed by analysis of variance.

Analyses of cells and growth medium during fermentation. Protein concentrations were determined with the bicinchoninic acid assay (42) with bovine serum albumin as a standard. Protein values are the averages from two independent samples, each measured twice. Cell density was monitored by measuring turbidity (OD_{578}) on an LKB 4057 UV-visible spectrophotometer. The specific growth rate was defined as $\mu = ln(x_2/x_1)/(t_2 - t_1)$, where x is OD_{578}, t is elapsed fermentation time (EFT), and subscripts 1 and 2 indicate different sampling times. The values for μ_e ($hours^{-1}$) mentioned in Results are the average values of μ during exponential growth phase. L-Malate and NH_4^+ concentrations in the supernatant were determined with test kits from Boehringer Mannheim (27). The O_2 concentration in the medium was measured by the Winkler method (Aquamerck oxygen test combination; Merck) (24).

Isolation of membranes. Bacterial cultures grown in an oxystat under aerobic, microaerobic NH_4^+-supplemented, and nitrogen-fixing conditions were harvested at the beginning of the stationary phase (OD_{578} of approximately 1.2). Cells were subsequently centrifuged and suspended in 3 ml of 25 mM TES [*N*-tris(hydroxymethyl)methyl)methyl-2-aminoethanesulfonic acid]-KOH–5 mM $MgCl_2$ buffer (pH 6.8) containing 10 μg of RNase per ml, 10 μg of DNase I per ml, and 1 mM phenylmethylsulfonyl fluoride. Membrane vesicles were prepared as described by Haaker et al. (16).

Visible difference absorbance spectra. Visible light spectra were recorded on a dual-wavelength scanning spectrophotometer (Aminco DW2). Scanning was performed from 400 to 700 nm with a 3-nm bandwidth and from 500 to 700 nm with a 1-nm bandwidth at a scan speed of 1 nm/s. For reduced-minus-oxidized spectra, the membranes were reduced with dithionite. For the CO plus dithionite-reduced-minus-dithionite-reduced difference spectra, dithionite-reduced membranes were sparged for 5 min with 100% CO. Measurements were taken after 15 min.

Recombinant DNA techniques. Standard protocols were used for cloning, restriction mapping, plasmid isolation, transformation, Southern blotting, and hybridization (36). Genomic DNA was isolated as described previously (2). PCR was performed on single colonies from *A. brasilense* Sp7. The primers used for the amplification of the *cytN* gene were cytplus (5′-TA**GAATTC**ARTGGTGGT AYGGNCAYAAYGC-3′) and cytminus (5′-CA**GAATTC**CRTTRATCATNCC SCCCCA-3′). Both primers were provided with *Eco*RI recognition sites (boldface) to facilitate cloning procedures. The PCR was carried out in a TRIO-thermoblock (Biometra) with 0.2 mM deoxynucleoside triphosphates, 1 μM each primer, and 0.025 U of *Taq* DNA polymerase (Boehringer) per μl. The following PCR protocol was used: a denaturation period of 6 min at 94°C; followed by 35 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C; followed by an extension reaction of 7 min at 72°C.

A 300-bp PCR fragment was cloned in the *Eco*RI site of the vector pEMBL18, and it revealed an open reading frame (ORF) whose deduced product had similarity to known *fixN* gene products. This 300-bp *Eco*RI insert was used as probe to screen a previously constructed genomic library of *A. brasilense* Sp7 in pLAFR1 (25). One hybridizing clone (pFAJ853) with an insert of approximately 16 kb was digested with *Kpn*I, and the 6-kb fragment hybridizing with the probe was subcloned in pUC18, resulting in pFAJ860 containing the entire *cytNOQP* operon.

The *Kpn*I fragment of pFAJ860 was further subcloned into pUC18 or pUCBM20 to obtain the overlapping fragments covering the entire *cytNOQP* operon (approximately 4 kb). All subclones were sequenced on both strands by the chain termination dideoxynucleoside triphosphate method (37) with the AutoRead Sequencing Kit (Pharmacia-LKB) on an automated sequencer (ALF; Pharmacia-LKB), using fluorescein-labeled universal and synthetic oligonucleotide primers. Sequence data were assembled and analyzed with the DNA-analyzing program PC-Gene (Intelligenetics). Sequence data banks were screened for similarities by using the BLAST program (1).

Mutant construction. To construct *cytN* insertion mutants, a 1.8-kb *Bam*HI fragment was subcloned into pUC18, resulting in plasmid pFAJ861. A 2.5-kb *aph*II cassette (encoding Kanamycin resistance [Km^r]) of pHP45Ω-Km was blunt ligated in the *Apa*I site of pFAJ861, resulting in plasmid pFAJ862 (Km^r cassette in the same orientation as *cytN* [plus direction]) and pFAJ863 (Km^r cassette in the orientation opposite that of *cytN* [minus direction]). The resulting fragment

was subsequently cloned as an *Eco*RI/*Xba*I fragment into the *Pst*I site of the suicide plasmid pSUP202 after blunting all sticky ends. These resulting plasmids, named pFAJ857 (plus direction) and pFAJ856 (minus direction) were subsequently mobilized from *E. coli* S17-1 into *A. brasilense* Sp7 by conjugation. Km^r *A. brasilense* exconjugants were screened for the loss of the recombinant plasmid and for double homologous recombination by replica plating on the appropriate antibiotics as Km^r and tetracycline-sensitive (Tc^s) clones. Recombination at the correct location was verified by Southern hybridization with DNA fragments from the *cytN* gene and the Km^r cassette as probes. The orientation of the cassette was verified by Southern hybridization. In FAJ851, transcription of the Km^r cassette is in the same orientation as that of the downstream genes *cytO* and *cytP*, while in FAJ852, transcription of the Km^r gene is opposite to the transcription orientation of the downstream genes.

SDS-PAGE and heme staining. Membrane proteins, isolated as described above, were subsequently dissolved in denaturing equilibration buffer (60 mM Tris-HCl [pH 6.8], 2% [wt/vol] sodium dodecyl sulfate [SDS], 10% [wt/vol] glycerol, 28 μM bromophenol blue, 5% [vol/vol] β-mercaptoethanol) and separated by polyacrylamide gel electrophoresis (PAGE) in SDS–15% (wt/vol) polyacrylamide gels (20). Protein samples were not heated before electrophoresis. The resulting gels were stained for covalently bound heme with *o*-dianisidine (12) before being stained with Coomassie blue.

Nucleotide sequence accession number. The sequence of the *cytNOQP* operon has been submitted to the GenBank/EMBL database under accession no. AF054871.

RESULTS

Spectral analysis of *A. brasilense* membranes. Membranes were isolated from cells as described in Materials and Methods. In the reduced-minus-oxidized spectra of membranes isolated from aerobically and microaerobically grown cells (Fig. 1A and C), the α peak at 552 nm and the β peak at 522 nm are attributable to *c*-type cytochromes and the 560-nm (β-peak) and 527-nm (β-peak) shoulders are attributable to cytochromes *b*. In membranes of microaerobically grown cells, the cytochrome *c* peak at 552 nm was clearly more pronounced than the cytochrome *b* shoulder at 560 nm (Fig. 1C), suggesting a relatively high cytochrome *c*/cytochrome *b* ratio. This high level of cytochrome *c* was also evident from the β band (522 nm), which showed asymmetry at shorter wavelengths (Fig. 1C). In the CO-binding spectrum reaction of CO with the high-spin heme is responsible for the inverted shoulder at 560 nm (Fig. 1B and D). Spectral analysis revealed that the terminal oxidases expressed in *Azospirillum* cells were similar in microaerobic conditions, whether or not combined nitrogen was available in the growth medium (data not shown). In aerobic conditions (Fig. 1C) the reduced-minus-oxidized spectrum showed a pronounced peak at 560 nm and a decreased peak at 552 nm. The CO-reduced-minus-reduced difference spectrum showed a clear inverted shoulder at 560 nm (Fig. 1D). These observations indicate a smaller amount of cytochrome *c* than of cytochrome *b* and suggest the presence of a second cytochrome *b*-containing oxidase present in aerobic conditions.

Analysis of the DNA sequence and the deduced amino acid sequences. Identification of the genes encoding this potential cytochrome cbb_3 oxidase was done by a PCR-based cloning procedure as described in Materials and Methods. The identified DNA fragment subcloned in pFAJ860 contained all of the genes of *A. brasilense* corresponding to known *fix*(*cyt*,*cco*) *NOQP* genes of other bacteria.

Four ORFs (*orf1*, *orf2*, *orf3*, and *orf4*) of, respectively, 1,494, 765, 159, and 885 bp were detected (Fig. 2). Each of these ORFs was preceded by a putative Shine-Dalgarno sequence upstream of the ATG start codon. *orf1* was preceded by a potential anaerobox (TTGA-N_5-ATCAA) 189 bp upstream of the ATG codon (9). At 60 bp downstream of *orf4*, a sequence with interrupted dyad symmetry (ΔG [25°C] = −25 kcal), followed by a T-rich region, suggests the presence of a Rho-independent transcription terminator (35). The amino acid sequences deduced from these ORFs showed high similarity with

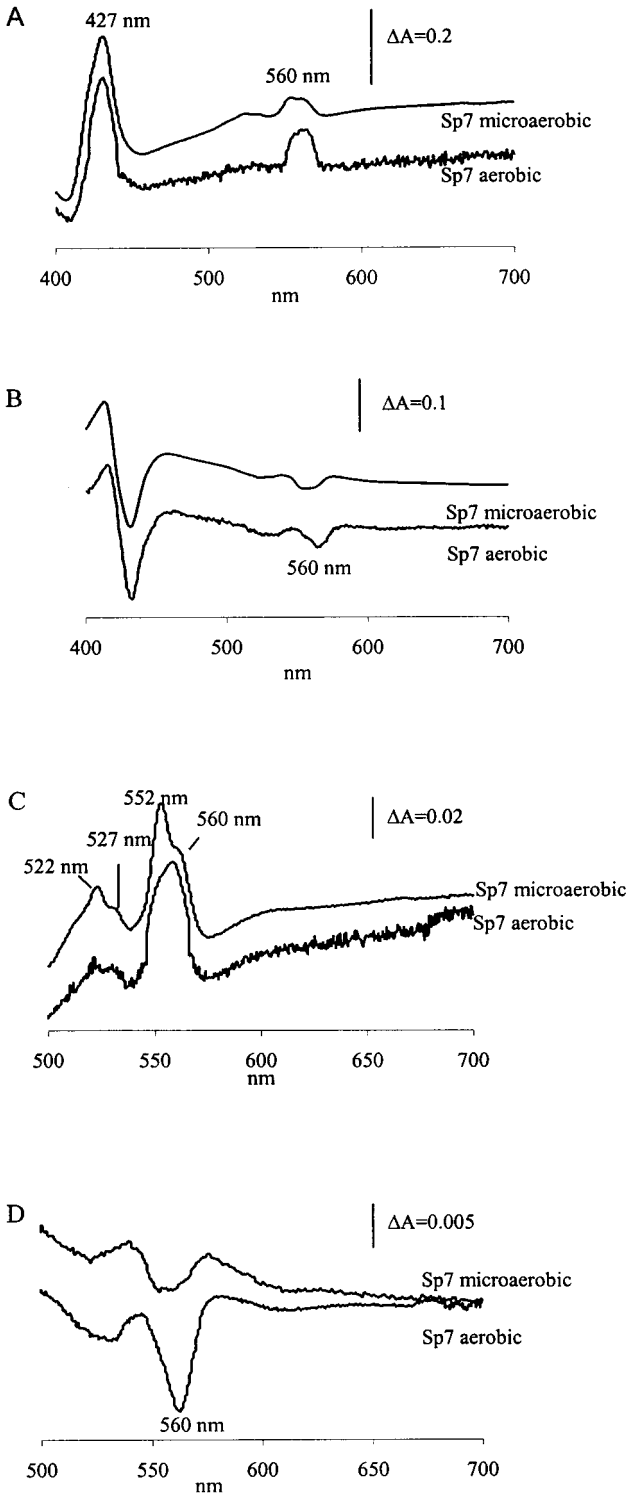

FIG. 1. Difference spectroscopy of membrane proteins isolated from microaerobically and aerobically grown *A. brasilense* Sp7. (A and C) Dithionite-reduced-minus-air-oxidized spectra between 400 and 700 nm (5 mg of protein/ml) (A) and between 500 and 700 nm (3 mg of protein/ml) (C). (B and D) CO plus dithionite-reduced-minus-dithionite-reduced difference spectra between 400 and 700 nm (2 mg of protein/ml) (B) and between 500 and 700 nm (2 mg of protein/ml) (D).

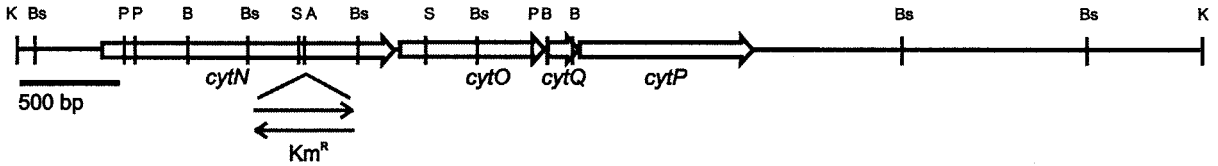

FIG. 2. Physical map of the 6-kb *Kpn*I fragment of pFAJ860. The 4-kb part containing the *cytNOQP* operon was completely sequenced on both strands. The region downstream of this 4-kb fragment was only partially sequenced. Both arrows indicate insertion of the Km^r cassette. Abbreviations: A, *Apa*I; B, *Bam*HI; Bs, *Bss*HII; K, *Kpn*I; P, *Pst*I; S, *Sal*I.

those of known genes, i.e., *fixN* (*cyt*,*ccoN*), *-O*, *-Q*, and *-P*. The identified ORFs were therefore designated *cytN* (*orf1*), *cytO* (*orf2*), *cytQ* (*orf3*), and *cytP* (*orf4*).

The *cytN* gene encodes a protein of 498 amino acids (predicted molecular mass of 56 kDa for the apoprotein). CytN of *A. brasilense* showed identities ranging from 68 to 70% with CytN-like proteins of *Rhodobacter sphaeroides* (accession no. U58092), *Sinorhizobium meliloti* (18) (accession no. X15079), *P. denitrificans* (7), *A. caulinodans* (23), *Bradyrhizobium japonicum* (32), and *R. capsulatus* (45).

cytO encodes an apoprotein of 246 amino acids with a predicted molecular mass of 27.7 kDa. An N-terminal transmembrane helix and a highly conserved heme *c*-binding site (CYNCH) at position 71 could be identified. CytO showed 62 to 68% identity with the aligned CytO-like proteins of *R. sphaeroides* (accession no. U58092), *S. meliloti* (18) (accession no. X15079), *P. denitrificans* (7), *A. caulinodans* (23), *B. japonicum* (32), and *R. capsulatus* (45).

cytQ encodes a small protein of 53 amino acids with a predicted molecular mass of 6 kDa. It exhibited identities of only 34% with the FixQ protein of *B. japonicum* (32), 40% with FixQ of *S. meliloti* (18) (accession no. X15079), 38% with CytQ of *A. caulinodans* (23), 34% with CcoQ of *R. capsulatus* (45), 30% with CcoQ of *R. sphaeroides* (accession no. U58092), and 20% with CcoQ of *P. denitrificans* (7). In *B. japonicum* FixQ is not involved in the assembly of the oxidase complex and seems not to be an essential subunit of the complex (55).

cytP codes for an apoprotein of 295 amino acids with a predicted molecular mass of 31.8 kDa. A hydrophobic stretch is located at positions 34 to 50. The protein exhibited two heme-binding motifs (CAACH at position 121 and CAACH at position 220). CytP showed an identity of 42 to 53% with the CytP-like proteins of *R. sphaeroides* (accession no. U58092), *S. meliloti* (18) (accession no. X15079), *P. denitrificans* (7), *A. caulinodans* (23), *B. japonicum* (32), and *R. capsulatus* (45).

Construction and phenotypic analysis of a *cytN* mutant. A Km^r insertion mutant was constructed as described in Materials and Methods. The Km^r cassette was inserted in both orientations (Fig. 2).

Membranes isolated from both *cytN* mutants and wild-type cells grown in microaerobic conditions were tested for the presence of covalently bound heme by SDS-PAGE followed by heme staining (Fig. 3). Six heme *c*-containing proteins, of approximately 6, 21, 27, 28, 32, and 40 kDa, were present in the wild type. In the *cytN* mutants FAJ851 and FAJ852, the 28- and 32-kDa heme-containing proteins, with molecular masses similar to the predicted molecular masses of *A. brasilense* CytP (31.8 kDa) and CytO (27.7 kDa), were absent. The other staining bands in both the wild type and *cytN* mutants represented other, yet-uncharacterized heme-containing proteins, such as NO reductase or bc_1 complex proteins present in *A. brasilense* cells grown under the tested conditions (17).

Reduced-minus-oxidized absorbance spectra of membranes isolated from the wild-type Sp7 and from mutants FAJ851 and FAJ852, grown to the beginning of the stationary phase in microaerobic batch cultures, are shown in Fig. 4. The relative high cytochrome *c*552/cytochrome *b*560 ratio, characteristic of the presence of a cytochrome cbb_3 terminal oxidase, was lowered in the mutants, as shown by the decrease in the cytochrome *c* peak at 552 nm. Furthermore, these spectra of membranes isolated from *A. brasilense cytN* mutants in microaerobic conditions showed a high similarity with spectra of membranes isolated from *A. brasilense* wild-type cells grown in aerobic conditions (Fig. 1C).

Growth of the *cytN* mutant and the wild type was compared under different conditions (Fig. 5). Specific growth rates during exponential phase (μ_e) were calculated as described in Materials and Methods. The *A. brasilense cytN* mutant and the wild type showed similar growth patterns, illustrated by the increase in OD_{578} (Fig. $5A_1$) and protein concentrations (Fig. $5A_2$), in aerobic conditions, although a higher protein concentration was obtained in wild-type cells. After an EFT of approximately 8 h, when the carbon source malate becomes limiting, cells entered the stationary phase (Fig. $5A_1$). The specific growth rates of *A. brasilense* Sp7 and the *cytN* mutant during exponential phase were similar (μ_e of approximately 0.2 h^{-1} [Fig. $5A_2$]).

In microaerobic conditions in a medium supplemented with NH_4^+, the low DO concentration had no influence on the growth behavior of the *A. brasilense* wild type compared to that in aerobic conditions (Fig. 5A and B). Within a few hours, the OD_{578} increased drastically (Fig. $5B_1$) (μ_e of approximately 0.2 h^{-1} [Fig. $5B_2$]), and the stationary phase was reached after an EFT similar to that for aerobically grown cells. In contrast, the specific growth rate of the *A. brasilense cytN* mutant during exponential phase was considerably affected (μ_e of approximately 0.02 h^{-1} [Fig. $5B_2$]). Only after an EFT of 40 h did the

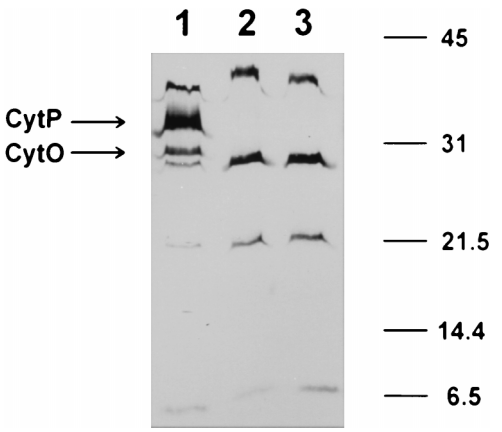

FIG. 3. Analysis for covalently bound heme in *A. brasilense* membrane proteins. Membranes were isolated from microaerobically grown wild-type *A. brasilense* (Sp7) (lane 1) and *cytN* mutants (FAJ851 [lane 2] and FAJ852 [lane 3]). Equal amounts of proteins (approximately 200 μg) were loaded. The positions of molecular size markers (Bio-Rad) are indicated by horizontal lines (in kilodaltons).

cells reach an OD_{578} similar to the OD_{578} obtained for the wild type at the beginning of the stationary phase (Fig. $5B_1$). The carbon source malate was still not entirely consumed. Respiratory behavior can be judged by the changes in the percentage of O_2 present in the incoming gas flow during fermentation. An increase in the percentage of O_2 is due to an increase in the airflow rate. The higher O_2 concentration in the incoming gas flow together with the simultaneously increased total gas flow through the fermentor causes a higher O_2 transfer rate, reflecting the higher O_2 consumption by the growing cells. Figure $5B_1$ shows that when the wild-type cells were grown in microaerobic NH_4^+-supplemented conditions, the percentage of O_2 in the gas flow, automatically adjusted to maintain a constant DO concentration of 2.5 μM, was increased at regular time intervals in order to cope with the high O_2 demand of the fast-growing cells. For the *A. brasilense cytN* mutant grown in similar conditions, a low constant percentage of O_2 in the gas flow was sufficient to maintain the DO concentration at 2.5 μM.

The specific growth rate of the wild type during exponential phase under nitrogen-fixing conditions (μ_e of approximately 0.03 h^{-1} [Fig. $5C_2$]) was decreased compared to specific growth rates obtained under the same conditions but in the presence of an NH_4^+ source (μ_e of approximately 0.2 h^{-1} [Fig. $5B_2$]). The cells needed approximately 30 h to reach stationary phase, and the final cell protein concentration was significantly lower than during NH_4^+-supplemented growth (Fig. $5C_1$ and C_2). The percentage of O_2 in the gas flow during fermentation remained relatively constant, indicating a low O_2 demand (Fig. $5C_1$). In these nitrogen-fixing conditions, the growth and respiratory behaviors of the *A. brasilense cytN* mutant did not differ drastically from those of the wild type (Fig. $5C_1$ and C_2). A lower rate of consumption of the carbon source malate, a slightly lower specific growth rate during exponential phase (μ_e of approximately 0.02 h^{-1}), and a reduction of the specific nitrogenase activity of the *A. brasilense cytN* mutant (13.5 ± 0.99 nmol of ethylene/mg of protein/h) to approximately 80% of the wild-type activity (16.52 ± 1.25 nmol of ethylene/mg of protein/h) were observed for the *A. brasilense cytN* mutant compared to the wild-type strain. The high specific growth rate observed for both the *A. brasilense cytN* mutant and the wild type at the start of nitrogen-fixing growth is probably due to the presence of internal NH_4^+ in the inoculated cells (Fig. $5C_2$).

DISCUSSION

The similarity between spectra shown in this work and those reported for the purified cbb_3-type cytochrome *c* oxidase complexes from *B. japonicum* (33), *R. capsulatus* (15), and *M. magnetoacticum* (43) suggests that an analogous cytochrome cbb_3 oxidase is present in microaerobically grown *Azospirillum* cells. Accordingly, and consistent with previous results, a relative increase in the level of cytochrome *c* versus cytochrome *b* was observed during a shift from aerobic to microaerobic conditions (6, 21).

Genetic evidence of a *cytNOQP* operon in *A. brasilense* supports this biochemical analysis. The *A. brasilense cytNOQP* operon is preceded by a putative anaerobox. So far, no direct evidence for the existence of an FNR-like protein in *Azospirillum* is available (47).

cytN of *A. brasilense* encodes subunit I of the cbb_3-type terminal oxidase. The highly conserved histidine residues shown to be involved in the binding of the high-spin *b*/Cu_B reaction center (5, 26) are conserved at positions 362, 274, 275, and 224 in the *A. brasilense* CytN. The histidine residues assumed to be the axial ligands for the low-spin heme *b* (22, 56) are located at positions 74 and 364. The histidine residue implicated in Mg^{2+} and Mn^{2+} binding in *B. japonicum* FixN (54) is present at position 354. The histidine residue suggested to bind and release protons in *B. japonicum* FixN (54) is at position 260. Based on a structural comparison between subunit I of conventional cytochrome *c* oxidases, containing 12 transmembrane helices, and the cytochrome cbb_3 oxidases, usually characterized by 14 potential transmembrane helices, Zufferey et al. (56) hypothesized that the first 2 of these 14 transmembrane helices of CytN-like proteins should be cytoplasmic. This hypothesis was supported by studies with fusion proteins (56). Interestingly the *A. brasilense* CytN protein seems to be truncated and lacks these two first transmembrane helices encountered in other sequenced CytN-like proteins.

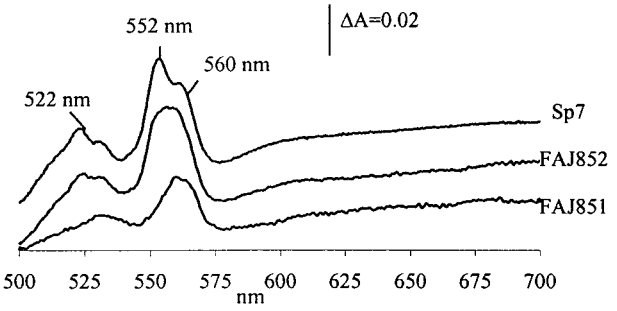

FIG. 4. Dithionite-reduced-minus-air-oxidized spectra, between 500 and 700 nm, of membrane proteins isolated from microaerobically grown cells of *A. brasilense* Sp7 (wild type) and FAJ851 and FAJ852 (*cytN* mutants). Equal amounts of protein (approximately 2.6 mg/ml) were analyzed.

To investigate the role of the cytochrome cbb_3 oxidase, a Km^r insertion mutant of *A. brasilense cytN* was constructed. Results from heme-stained SDS-PAGE gels and spectral analysis of membranes from both *A. brasilense* wild-type and *A. brasilense cytN* mutant cells led us to conclude that the *A. brasilense cytN* mutant lacks a functional cytochrome cbb_3 terminal oxidase.

Subsequently, growth analysis was performed. In microaerobic conditions a high respiration rate potentially supporting efficient energy production allows the *A. brasilense* wild-type cells to grow at rates similar to those obtained in highly aerated cultures, despite the low DO concentration. As the *A. brasilense cytN* mutant was not able to sustain such growth, the cytochrome cbb_3 oxidase seems to be responsible for the high respiration rates observed at low DO concentrations. During nitrogen fixation, the specific growth rate of the wild type was considerably lower than in NH_4^+-supplemented conditions. As nitrogen fixation is a very energy-consuming process, a shortage of ATP seems a plausible cause for growth limitation. This seems to be the case in symbiotic microorganisms such as *Rhizobium* or *Bradyrhizobium* species, where nitrogen fixation takes place in nodules. These nodules create the optimal low O_2 concentration to prevent O_2 damage to the nitrogenase and function simultaneously as an O_2 delivery system (40) to a high-affinity cytochrome cbb_3 terminal oxidase. This oxidase allows high respiration rates and generation of a proton motive force at nanomolar concentrations of O_2 (33). Cytochrome cbb_3 mutants are completely (32) or at least partially (19, 23) unable to fix N_2, indicating the importance of energy as a limiting factor. Assuming that energy limitation explains the lower specific growth rates of the wild-type *A. brasilense* during nitrogen fixation, the *A. brasilense cytN* mutant affected in its cytochrome cbb_3 terminal oxidase would be expected to show an even more pronounced energy-limited growth. However, only minor differences were observed between the specific

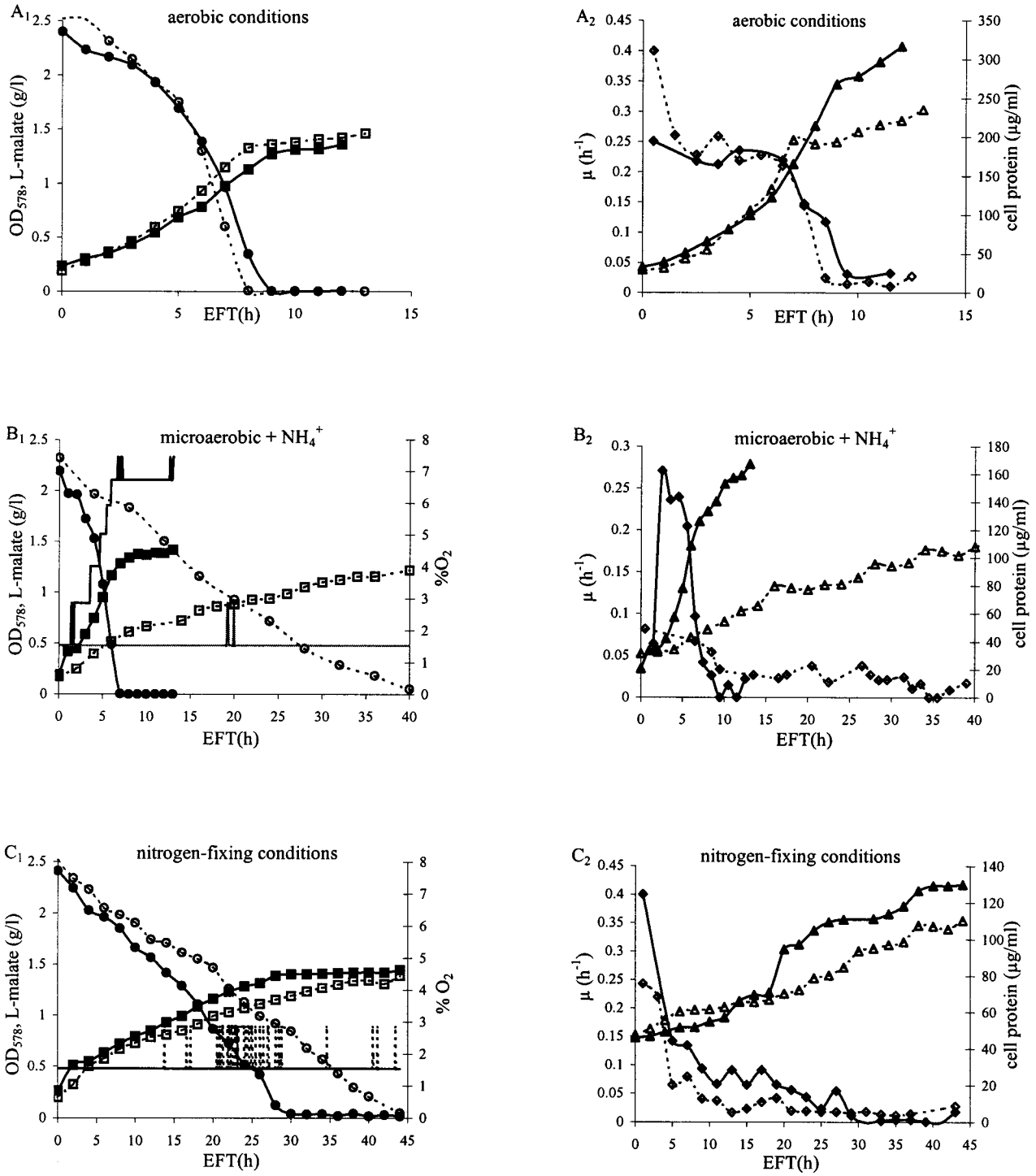

FIG. 5. Comparison of fermentation parameters of the wild-type *A. brasilense* Sp7 and the *A. brasilense cytN* mutant FAJ851 under aerobic (A), microaerobic and NH_4^+-supplemented (B), and nitrogen-fixing (C) conditions. (A_1, B_1, and C_1) OD_{578} values and malate concentrations. Panels B_1 and C_1 also show the percentage of O_2 present in the incoming airflow during fermentation in microaerobic conditions. Note that in panel C_1 the lines for percent O_2 during fermentation of the wild-type Sp7 and the *cytN* mutant FAJ851 coincide. (A_2, B_2, and C_2) Protein concentrations and specific growth rates (μ). Values for protein concentration and L-malate are the averages of at least four different measurements. (A_1, B_1, and C_1) ■, OD_{578} for Sp7; □, OD_{578} for FAJ851; ●, L-malate for Sp7; ○, L-malate for FAJ851; —, percent O_2 for Sp7; ----, percent O_2 for FAJ851. (A_2, B_2, and C_2) ▲, protein for Sp7; △, protein for FAJ851; ◆, μ for Sp7; ◇, μ for FAJ851.

growth rates of the *A. brasilense cytN* mutant and the wild type in these conditions, and the nitrogenase of the *A. brasilense cytN* mutant still retained approximately 80% of its activity. Therefore, a more likely explanation of growth limitation in nitrogen-fixing conditions seems to be the shortage of NH_4^+. Possibly the nitrogenase cannot produce sufficient NH_4^+ to cope with the high NH_4^+ consumption by fast-growing cells. Alternatively, the strict regulation of the nitrogen-fixing process cannot be excluded as a growth-limiting factor. If cells fix nitrogen at high rates, the internal NH_4^+ accumulating in the

cells could switch off the system (52). Nitrogen fixation and thus the NH_4^+ concentration consequently decrease, which in turn allows the system to resume nitrogen fixation. Conceivably, there can never be an accumulation of sufficient NH_4^+ to allow fast growth and subsequent energy limitation.

Similar to the observations made for *A. caulinodans* (19, 23), an unknown alternative oxidase can partially overcome the absence of the cytochrome cbb_3 terminal oxidase in microaerobic conditions, either in presence or absence of NH_4^+, since *A. brasilense cytN* mutants could still grow. Similar to previous results for *A. brasilense* Sp7 (6), but in contrast to those for *A. brasilense* Cd (34), no terminal oxidase containing cytochrome *d* seems to be present. No indications could be found for the presence of heme *a*, not even in membranes of aerobically grown cells. Although the concentrations of heme *a* discovered before in *A. brasilense* Sp7 were barely detectable (21), it cannot be ruled out that under certain conditions this cytochrome *c* oxidase is expressed. Comparison of the reduced-minus-oxidized spectra of membranes from the *A. brasilense cytN* mutant and the wild type points in the direction of an additional heme *b*-containing terminal oxidase such as, e.g., a *bo*-quinol oxidase during microaerobic growth. This oxidase also seems to be present in fully aerated membranes of the wild type. A conclusive interpretation of the CO-reduced-minus-reduced spectra (data not shown) was hampered by interference of the absorption maxima of this potential *bo*-quinol complex by the absorption maxima of other proteins putatively present in the membranes, such as the bc_1 complex, denitrifying complexes, or even other alternative oxidases (11). However, the presence of a *bo*-quinol oxidase seems to be consistent with earlier reports on *A. brasilense* Sp7, which indicate the presence of particulate cytochrome *b* (6, 21) and a CO-binding cytochrome *o* (6, 21) in aerobic conditions. Likewise, spectral analysis suggested the presence of a cytochrome *o*-containing terminal oxidase in *A. brasilense* Cd, expressed in aerobic but also in microaerobic conditions (34). This terminal oxidase, however, seemed to function after the antimycin A inhibition site (after the cytochrome *c* reductase complex). In addition it was shown that an alternative oxidase, other than cytochrome caa_3-type cytochrome *c* oxidase and less sensitive to KCN, could accept electrons from TMPD (*N*,*N*,*N*′-tetramethyl-*p*-phenylenediamine) plus ascorbate, indicating the presence of another cytochrome *c*-type terminal oxidase (34). We suggest that, given the spectral similarities between cytochrome *o*-containing and cbb_3-type terminal oxidases, the cytochrome *o*-like cytochrome *c* oxidase identified previously is identical to the cytochrome cbb_3 cytochrome *c* terminal oxidase of *A. brasilense* Sp7 characterized in this study. The presence of such a cbb_3-type cytochrome *c* oxidase might have accounted for the residual reduction of ascorbate in the presence of a low concentration KCN, which is known to inhibit the cytochrome caa_3-type cytochrome *c* oxidase.

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