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Oxygen-Dependent Transcriptional Regulation of Cytochrome aa_3 in *Bradyrhizobium japonicum*

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Cytochrome aa_3 is one of two terminal oxidases expressed in free-living *Bradyrhizobium japonicum* but not symbiotically in bacteroids. Difference spectra (dithionite reduced minus ferricyanide oxidized) for membranes from cells incubated with progressively lower O_2 concentrations showed a concomitant decrease in the A_{603}, the absorption peak characteristic of cytochrome aa_3. The level of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine oxidase activity, a measure of cytochrome aa_3 activity, was also found to depend on the O_2 level. Dot blots of total RNA isolated from cells grown at various O_2 levels were probed with a fragment of the *coxA* gene from *B. japonicum*; a sixfold reduction in transcription from the highest (250 μM) to the lowest (12.5 μM) O_2 concentration was observed. Bacteroids had even less *coxA* message, approximately 19% that in the 12.5 μM O_2-incubated cells. Primer extension analysis established the transcription initiation site of the *coxA* gene at 72 bases upstream of the putative translational start codon. Sequence analysis of the region upstream of the transcription initiation site revealed no homology with previously reported *B. japonicum* promoters.

Cytochrome aa_3 (cytochrome *c* oxidase; EC 1.9.3.1) is a terminal oxidase that catalyzes the reduction of molecular oxygen to water while oxidizing cytochrome *c*. The enzyme is the sole terminal oxidase found in the mitochondria of eukaryotic cells and is nearly ubiquitous in aerobic bacteria (14). This enzyme has been the subject of intense study for several decades in eukaryotes and more recently in several prokaryotic systems (1). Much of the attention has been focused on the biochemical and physical properties of the enzyme, and only recently have the molecular mechanisms involved in the genetic regulation of the enzyme been addressed. Genes encoding structural subunits of cytochrome aa_3 have been cloned and sequenced for several prokaryotic organisms, including *Paracoccus denitrificans* (34), *Bacillus subtilis* (30), *Rhodobacter sphaeroides* (31), PS3 (13), and *Bradyrhizobium japonicum* (7, 10).

In most prokaryotes, cytochrome aa_3 is expressed along with a second terminal oxidase. This situation is also the case for *B. japonicum*, in which cytochrome aa_3 is the terminal oxidase expressed at the end of the low-O_2-affinity branch of the electron transport pathway, the other being cytochrome *o* (26). Cytochrome aa_3 is expressed at high levels in fully aerobic, free-living cultures (3). Under conditions in which O_2 levels are microaerobic ($O_2 << 1\%$), e.g., in bacteroids isolated from root nodules, cytochrome aa_3 is not detectable (2). While this situation is generally the case for both *Bradyrhizobium* and *Rhizobium* species, there are species and specific strains that do retain cytochrome aa_3 under symbiotic conditions (15). This fact may indicate that there are additional factors other than environmental O_2 that may regulate cytochrome aa_3 expression in rhizobia.

In this study, we present evidence that *coxA* (7, 10), encoding the largest structural subunit of the cytochrome aa_3 enzyme complex in *B. japonicum*, is regulated at the transcriptional level by O_2.

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MATERIALS AND METHODS

Chemicals and reagents. All chemicals were reagent grade or better and were obtained from Sigma Chemical Co. (St. Louis, Mo.) or J. T. Baker Chemical Co. (Phillipsburg, N.J.). All gases were purchased from Potomac Airgas (Baltimore, Md.). [γ-^{32}P]ATP and [α-^{32}P]dCTP were purchased from New England Nuclear. Moloney murine leukemia virus reverse transcriptase was purchased from Stratagene (La Jolla, Calif.).

Bacterial strains and growth. *B. japonicum* LO is a nalidixic acid-resistant derivative of strain USDA DES 122 and was the wild-type strain used for this study. *B. japonicum* LO was routinely grown in MB medium (24) at 29°C. For incubation with reduced O_2, strain LO was shifted to medium lacking carbon as described previously (33) after being grown to the mid-log phase in MB medium. *B. japonicum* grows chemoautotrophically and respires very slowly in this type of medium (18, 33). We chose this medium to minimize the effects of respiration on dissolved O_2 levels. After extensive sparging with a non-O_2-containing gas mixture composed of 85% N_2, 10% H_2, and 5% CO_2, O_2 was added in various amounts and the cells were incubated at 29°C with shaking at 180 rpm for 18 to 24 h. These incubations were carried out in gas-tight stoppered 6-liter flasks. The culture volume in these flasks was kept at 0.5 liter to maximize the headspace/liquid medium ratio.

Cytochrome spectra and heme contents. Cytochrome spectra of membranes were determined on a Hitachi-Perkin Elmer model 557 spectrophotometer as previously described (24). Heme contents of membranes were determined by using the extinction coefficients determined by Appleby (2, 3).

Membrane preparation. Cultured cells or bacteroids were broken by two passes through a French pressure cell at 1,440 kg/cm^2 (21). Centrifugation was done at low speed to remove unbroken cells and debris. Membranes were then pelleted by centrifugation at 110,000 × *g* for 1 h (24). Membranes were resuspended at the appropriate protein concentrations in 50 mM potassium phosphate buffer (pH 7.0) containing 2.5 mM $MgCl_2$. All manipulations were performed at 4°C.

Protein determination. Protein concentrations were determined with the bicinchoninic acid protein assay reagent from Pierce (Rockford, Ill.) and bovine serum albumin as the standard.

RNA isolation. Total RNA was isolated essentially as described previously (17) with minor modifications (12).

Identification of the *coxA* transcription start site. Primer extension reactions were performed exactly as described previously (5). The primer used was a 26-base oligonucleotide.

Growth of soybeans and isolation of bacteroids from root nodules. Soybeans (*Glycine max* bv. Essex) were grown in vermiculite supplemented with nitrogen-free nutrients in a greenhouse as described previously (35). The plants were grown under natural light and supplemental light (19) in Baltimore, Md., during the early summer of 1992. Seedlings were germinated and inoculated as described previously (24). Nodules were harvested 34 to 37 days after planting and were immediately stored at −70°C. Bacteroids were isolated from nodules as described previously (23).

Respiration assays. Assays were performed at room temperature in 100 mM phosphate buffer. Substrate oxidation was performed with cell extracts and ascorbate–*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) as the substrate (23). Concentrations were 10 mM ascorbate and 150 μM TMPD in phosphate buffer with 5 mM EDTA. Oxygen consumption was monitored amperometrically as described previously (20).

Quantitation of the *coxA* message. *coxA* message levels were determined by using both slot and dot blots as described previously (29). Message levels were quantitated by using either scanning densitometry of the autoradiograms of the blots or a Molecular Dynamics Phosphor Imager. Equivalent results were produced by both methods.

RESULTS

Effect of O_2 on hemoprotein content and cytochrome aa_3 oxidase activity. Difference absorption spectra (dithionite reduced minus potassium ferricyanide oxidized) for *B. japonicum* membranes from cells incubated with progressively decreasing O_2 levels showed a decrease in the level of spectrophotometrically detectable heme *a* (Fig. 1). Heme contents of the membranes were determined on the basis of the characteristic α peaks of the cytochrome spectra and the known extinction coefficients (2, 3). The levels of heme for O_2 levels of 20, 15, 10, 5, and 1% were 401, 173, 140, 140, and 137 nmol/g of protein, respectively. No heme *a* was spectrophotometrically detectable in bacteroid membranes. Along with the decrease in the heme *a* level with decreasing O_2 levels, concomitant increases in the levels of both heme *b* and heme *c* were observed (Fig. 1). It is interesting to note that although heme *b* and *c* levels increased with decreasing O_2 levels, the ratios of heme *b* and heme *c* remained relatively stable (data not shown).

Cytochrome aa_3 enzyme activity was measured by using TMPD as an artificial electron donor and measuring the rate of O_2 consumption amperometrically. TMPD oxidation paralleled the spectrophotometric levels of detectable heme *a* closely (Fig. 2). In cells grown with high O_2 levels, in which cytochrome aa_3 is fully expressed, as determined by spectral studies, TMPD oxidase activity was also at its highest level, and the activity decreased with decreasing O_2 levels. In support of the TMPD oxidase assay as a measure of cytochrome aa_3 expression, a Tn*5*-induced cytochrome aa_3 insertion mutant of *B. japonicum* (23, 27) failed to oxidize TMPD (23). Although no heme *a* was detectable in bacteroid membranes, cell extracts from bacteroids showed a significant level of TMPD oxidase activity (data not shown). This result indicates that an oxidase other than cytochrome aa_3 oxidase can oxidize TMPD in bacteroids, as previously proposed (25).

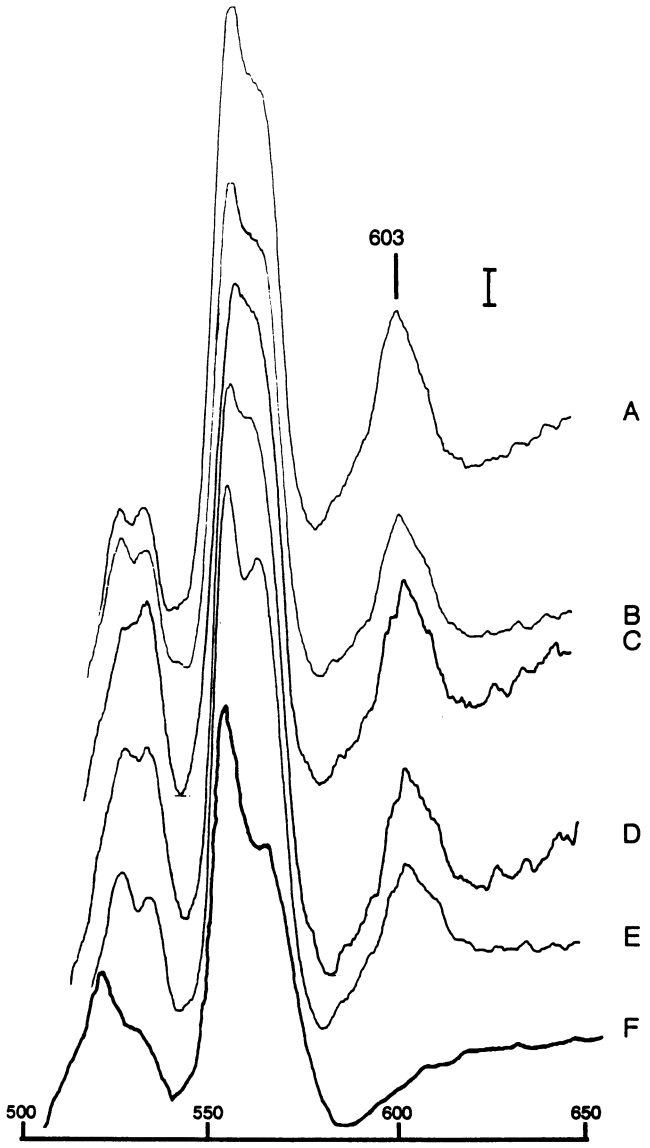

FIG. 1. Difference absorption spectra (reduced minus oxidized) for *B. japonicum* membranes. Cells were grown and membranes were treated as stated in Materials and Methods. Samples were membranes from cells grown with different O_2 levels: 20% (A), 15% (B), 10% (C), 5% (D), 1% (E). Trace F shows bacteroids. The protein concentration was 3 mg/ml, except in A and F, in which it was 2 mg/ml. The scale bar represents a change in the absorption of 0.001. Numbers on the abscissa are wavelengths, in nanometers.

Determination of the transcription initiation site and the *coxA* promoter. The *coxA* gene, encoding the largest of the structural subunits of cytochrome aa_3 in *B. japonicum*, was previously cloned and sequenced (7, 10, 27). Using primer extension analysis, we determined the transcription initiation site of the *coxA* gene. A 26-base oligonucleotide primer

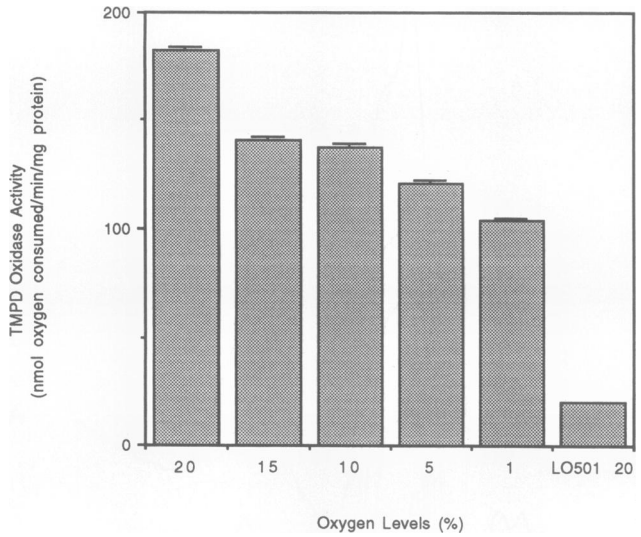

FIG. 2. TMPD oxidase activity in *B. japonicum* cell extracts from cells incubated with different O_2 levels. The concentrations of the substrates were 10 mM ascorbate and 150 μM TMPD. Results shown represent the activities in cell extracts, and error bars represent the standard deviations for five trials. The sample designated LO501 20 represents the TMPD oxidase measurements for cell extracts from a Tn*5*-induced cytochrome aa_3 mutant incubated with 20% O_2.

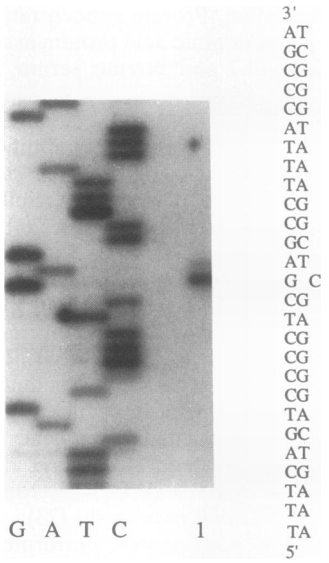

FIG. 3. Determination of the transcription initiation site of the *coxA* transcript by primer extension. A ^{32}P-end-labeled oligonucleotide primer was annealed to 10 μg of total cellular RNA and extended with Moloney murine leukemia virus reverse transcriptase. The DNA product was electrophoresed on a 6% denaturing polyacrylamide gel. The mobility of the extended product (lane 1) was compared with that of a dideoxy sequencing ladder (lanes G, A, T, and C) produced with the same primer and the sense strand of a *coxA* subclone as a template. The complement of the nucleotide that corresponds to the transcription start site of the *coxA* mRNA is offset.

which has the sequence 5′ GGTCTTGCGCGTGATCGC CGTGTGCC 3′ and which maps to nucleotides 116 to 90 in the *coxA* open reading frame was labeled at its 5′ end, annealed to total RNA isolated from free-living cells, and extended by using Moloney murine leukemia virus reverse transcriptase. The primer extension products were then electrophoresed, and dideoxynucleotide sequencing reactions were carried out by using the same primer (Fig. 3). The 5′ terminus of the *coxA* mRNA was located 72 bases upstream of the putative translation initiation codon (Fig. 4). Total RNAs isolated under all growth conditions showed the same transcription start sites (data not shown).

Figure 4 shows the sequence upstream of the putative transcription initiation site of *coxA*. Sequence analysis and comparison of this region with other known *B. japonicum* promoters revealed no homology in the σ factor binding region ranging from −10 to −35 or any region further upstream. A comparison with other *coxA* promoters in other organisms also revealed no homology.

Effect of O_2 on the transcription of *coxA*. We monitored the expression of *coxA* message levels in response to O_2 levels by using dot blots. Ten micrograms of total RNA isolated from *B. japonicum* cells grown with six different O_2 levels was used for dot blots. The blots were probed with a 1.2-kb ^{32}P-labeled fragment of the *coxA* structural gene (10, 27). The signal from the blots was quantitated, and typical results are shown in Fig. 5. As expected, the level of expression of the *coxA* message was highest in cells grown with 20% O_2 and decreased significantly with decreasing O_2 levels. The levels of the message expressed as a percentage of the levels in fully aerobically grown cells were 82, 79, 45, 18, and 3% for cells incubated with 15, 10, 5, and 1% O_2 and for bacteroids, respectively. Again, decreased levels of expression, in this case at the message level, correlated with decreasing O_2 levels. Surprisingly, total RNA isolated from bacteroids also produced a signal, indicating that *coxA* was transcribed at very low levels in the bacteroids, even though no spectrophotometrically detectable cytochrome aa_3 was present. The control blot, with 10 μg of yeast tRNA, showed no signal.

DISCUSSION

By using both spectrophotometry and enzyme activity measurements, we showed that cytochrome aa_3 was regulated by O_2. Quantitative dot blot analysis with the *coxA* gene as an indicator showed that this regulation occurs at the transcriptional level. There was a sixfold reduction in *coxA* message levels between the free-living, fully aerobically grown cells and cells grown with 1% O_2. Interestingly and surprisingly, some *coxA* message was also present in bacteroid cells but at very low levels. However, there was no

GAATTCGCCTCCTGGGTCGAAACGGCGAAGAAGAAGTTTGCGAGCGGTGGC

CACCGGCACCTACGCCTTCCGCGGCCGGCGACGCAGTAGGCGTCGGGTAAA

*

GGCTCGAGGGGACTGAAAGCGACATAAAGGGGCGGGACCTCCAAAGGTCCG

ATTGGGACGCAAGGCAGGATTTGAAA**ATG**GCAACCAGCGCAGCGGCACACG

FIG. 4. Nucleotide sequence of the *coxA* upstream region. The putative transcription initiation site is indicated by an asterisk. The presumed −10 to −35 region is underlined. The putative ribosome binding site is overlined. The proposed translation start codon is shown in boldface type.

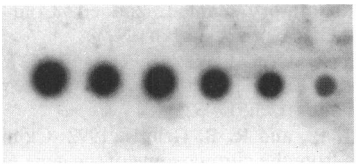

FIG. 5. Quantitation of *coxA* mRNA expression with various O_2 levels. Ten micrograms of total RNA for each of the culture conditions was blotted onto nitrocellulose filter paper and probed with a 1.2-kb ^{32}P-labeled fragment of the *B. japonicum coxA* gene. Samples were grown with the following O_2 levels: 20% (A), 15% (B), 10% (C), 5% (D), and 1% (E). Sample F was bacteroids. Signal levels were quantitated by using both a Molecular Dynamics Phosphor Imager and a Molecular Dynamics scanning densitometer.

spectrophotometrically discernible cytochrome aa_3, as seen by others (2, 15).

The start site of the *coxA* message was determined by using primer extension analysis, and the putative promoter area was identified. Most of the genes isolated from *B. japonicum*, whose promoters have been studied, are in some way involved with nitrogen metabolism (32) or are induced during bacterium-host plant interactions (32). Genes that are induced under low-O_2 conditions most often have a σ^{54} type consensus binding site. Since no constitutively expressed genes or genes repressed under low-O_2 conditions have been analyzed to date, no other canonical promoter sequences from *B. japonicum* are known. Perhaps the *coxA* promoter has a typical promoter representative of all promoters for genes expressed in free-living cells. *coxA* is in fact the first example of an O_2-responsive, transcriptionally down-regulated gene identified in *B. japonicum*. The fact that some transcription occurs in bacteroid cells may be attributed to two possible factors: (i) there exists a basal level of transcription in bacteroid cells, even though the enzyme encoded by the gene is not expressed, or (ii) the message detected is actually due to a message still being synthesized by undifferentiated bacteria that have not yet differentiated into mature bacteroids.

The possible reasons why cytochrome aa_3 is retained in the bacteroids of some *B. japonicum* strains (15), in which it is dispensable or nonessential (16, 22), have been the subject of speculation (28). The presence of cytochrome aa_3 expressed symbiotically in some strains may be due to a lack of tight O_2-dependent regulation in these strains.

Low O_2 levels in bacteroids are necessitated by the extreme oxygen lability of the nitrogenase enzyme complex. Low O_2 levels surrounding bacteroids are maintained, in part, by leghemoglobin, which functions in an O_2-buffering capacity (6, 8, 36).

It has been noted that the transition from free-living cultured cells to bacteroids is accompanied by a change in the compositions of terminal oxidases present in the cells. The most obvious environmental change in the transition from fully aerobic cultured cells to bacteroids is the profound decrease in free O_2 levels (4, 6, 8). Since free O_2 levels are so low under symbiotic conditions, estimated at 11 nM (4), and since the cytochrome aa_3 terminal oxidase has a relatively low O_2 affinity in both *B. japonicum* (28) and other prokaryotic systems (9, 11, 37) (an apparent O_2 affinity of 4 to 7 μM), cytochrome aa_3 may not be physiologically active at very low O_2 concentrations, and transcription will thus be repressed.

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