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Roles of the *Bradyrhizobium japonicum* terminal oxidase complexes in microaerobic H_2-dependent growth

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Abstract

Spectral, inhibitor, and O_2-consumption studies on membranes from free-living and bacteroid forms of *Bradyrhizobium japonicum* have revealed the existence of a number of terminal oxidases, and four terminal oxidase gene clusters within the heme–copper cytochrome family have been cloned. Here the complexes encoded by *cox*MNOP and *cox*WXYZ, genes with homology to Cu_A-containing cytochrome *c* oxidases and *b*-type ubiquinol oxidases respectively, are studied by analysis of mutants in each of the two oxidases and a double mutant in both of the terminal oxidase genes. Membranes from microaerobically incubated strain JHK12 (which contains an insertion in *cox*WXYZ) were deficient in levels of CO-reactive heme *b*, and both strains JHK12 and Bj3430 (the latter lacks *cox*MNOP) were deficient in CN^--reactive cytochrome *b*. Membranes of the double mutant (strain JHKS4) retained less than 7% of the cytochrome b_3 and 25% of the total CN^--reactive cytochrome *b* of the wild type. Cyanide inhibition curves of oxygen uptake by wild-type membranes were triphasic, and only the phases inhibited by the highest (at about 50 μM CN^-, attributed to cytochrome aa_3) and the lowest (at approximately 0.1 μM) CN^- were identifiable in the membranes from the two individual oxidase mutants. Membrane respiratory activity of the double mutant was resistant to CN^- over a broad inhibitor concentration in the micromolar range. Consistent with our findings that these oxidases are expressed when cells are incubated in a low O_2 environment, the double mutant was severely deficient in H_2-dependent chemolithotrophic growth. The latter growth condition requires prolonged incubation in an atmosphere of H_2, CO_2, and a low (1% or less) partial pressure of oxygen. The double mutant was also deficient in whole cell O_2 dependent H_2 oxidation, with H_2 uptake rates 31% of the wild type. © 1998 Elsevier Science B.V.

Keywords: Terminal oxidase complexe; Heme–copper cytochrome family; *(Bradyrhizobium japonicum)*

1. Introduction

Bradyrhizobium japonicum encounters and utilizes a broad range of oxygen levels. This is because the bacterium exists both as an aerobic free-living soil organism and as a bacteroid that fixes N_2 under very low oxygen tensions. The bacteroid is nevertheless an obligate aerobe, respiring O_2 in the low-O_2 environ-

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ment of the legume root nodule [1]. In symbiosis, it is incumbent upon the vigorously respiring bacterium to generate the ATP and reductant necessary to supply the energy-intensive nitrogen fixation process. The oxygen levels encountered by the free-living bacterium range up to 20% partial pressure (approximately 250 μM) O_2, whereas the free O_2 concentration within root nodules but surrounding the bacteroid achieve nM levels [2,3]. In order to accommodate this range of free oxygen concentrations, *B. japonicum* has a multiple-branched electron transport chain, with each branch terminating at an oxidase with a presumably different affinity for oxygen [4–7]. In addition to this symbiotic respiratory complexity, the free-living (*B. japonicum*) can grow in O_2 tensions ranging from air to microaerobic conditions. For the latter growth (chemolithotrophy), H_2 is used as reductant and CO_2 is the sole carbon source (see Ref. [8]).

Spectral and inhibition studies on membranes isolated from free-living and bacteroid forms of *B. japonicum* have revealed the existence of a number of terminal oxidases. These include an aa_3-type cytochrome *c* oxidase, a heme *b*-containing ubiquinol oxidase, a high O_2 affinity cytochrome *c* oxidase, and an unusual putative flavoprotein oxidase [8,9]. A cytochrome *c* oxidase complex of 7–8 subunits, containing a CO-reactive cytochrome *c* and capable of functioning at O_2 concentrations of less than 1.0 μM, was purified from *B. japonicum* bacteroid membranes [10]. Most of the subunits of this complex correspond to those encoded by the *fix*NOQP gene cluster that encodes a bb_3-type cytochrome *c* oxidase also of high O_2 affinity [7].

Gene clusters for four terminal oxidases in the heme–copper cytochrome family [11] have been cloned. These are *fix*NOQP [12], *cox*MNOP [13], *cox*BA [14,15,7] and *cox*WXYZ [16,17]. *cox*BA encodes the subunits of the cytochrome aa_3, and is expressed only in conditions of high aeration [18,19]. *cox*MNOP and *cox*WXYZ encode complexes with homology to Cu_A-containing cytochrome *c* oxidases [13] and *b*-type ubiquinol oxidases [20], respectively. Based on the predicted properties of CoxWXYZ and due to the lack of detectable heme O in *B. japonicum* [20] it was concluded the CoxWXYZ complex is a bb_3 type ubiquinol oxidase.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of reagent or molecular biology grade and were purchased from Sigma (St. Louis, MO), J.T. Baker (Phillipsburg, NJ), Amresco (Solon, OH), or Research Organics (Cleveland, OH). Bacterial growth media were obtained from Difco Laboratories (Detroit, MI). Trace elements used to make MB and no-carbon media were purchased from Morton Thiokol, Alfa Products (Danville, MA). All gases were purchased from WSC Specialty Gases (Baltimore, MD).

2.2. Bacterial strains and culture conditions

Strain JH is a USDA 110 derivative [21] and strains JHK12 [20], LO501 [9] and Bj3430 [13] have all been described previously. The double mutant strain (JHKS4) was made as described for strain JHK12 [20] except that *E. coli* containing pMA2 was mated with Bj3430 (*cox*MNOP mutant) to create JHKS4. Southern analysis confirmed that the kan^r cassette is integrated at the *cox*X locus (data not shown). All are derivatives of the isogenic strain USDA 110. *B. japonicum* cultures were grown on a shaker (150 rpm) at 30°C in MB media [22] until the O.D. (A_{540}) of the culture was 0.7–1.0. The tests for chemolithotrophic growth in 1% O_2, or for heterotrophic growth in less than 20% partial pressure O_2 were performed as described previously [23], and A_{540} measurements taken or viable cell number determined on MB plates. The mineral salts medium described previously [24] without a carbon source but with ammonium chloride (0.15 g/l) and nickel chloride (5 μM) added was used for chemolithotrophic growth [23]. This was done with 60 ml medium in 250 cc stoppered bottles in a gas phase of 10% H_2, 5% CO_2, 1% O_2, (balance N_2) and was readjusted to this composition every other day. The bottles were shaken at 100 cycles/min at 30°C. The inoculum was from cells previously incubated in this chemolithotrophic condition, and the fresh media (previously sparged with the $H_2/CO_2/N_2$ gas mixture) was inoculated to a beginning cell number of approximately 5×10^7 cells per ml (OD_{540} of approx-

imately 0.05). The first sample (day 1) was taken 24 h after this experimental set-up. Samples for OD measurements were removed aseptically (every other day for chemolithotrophic growth determinations) from the stoppered serum bottles by use of a syringe and needle so as not to disturb the gas atmosphere in the bottle. The bottles were re-sparged with the H_2, CO_2 and N_2 gas mixture every other day, and O_2 injected again to 1% partial pressure. For adapting cells to the microaerobic condition, which is a critical requirement for observing the expression of the oxidases studied, cultures were first grown in MB media to an O.D. (540 nm) of approximately 0.7–0.8. The bacterial cells were then collected via centrifugation (6000 rpm, 4°C, 15 min), and the pellets were resuspended in a mineral salts medium previously described [24] but lacking an organic carbon source. The cells were incubated shaking at 100 rpm (2 l of medium in a 6 l flask) under a gas-tight atmosphere composed to 10% H_2, 5% CO_2, 1% O_2, 84% N_2 for three days at 30°C. This is referred to as the 'microaerobic incubation' procedure.

2.3. Isolation of membranes

Cells from microaerobic incubation were collected via centrifugation ($6000 \times g$, 15 min 4°C). The pellets from 6 l of cells were taken up in 10 ml of ice-cold 0.05 M phosphate buffer. Butylated hydroxytoluene (4 mg/ml) was added to the resuspended cells to prevent oxidation of the membrane lipids, which results in a greater yield of bacteroid oxidase activity [25]. The membranes were then isolated as previously described [26]. Membranes were either used immediately, or stored (under an 100% Ar atmosphere) on ice for up to several hours. Total membrane protein was measured using the BCA protein assay from Pierce Chemical (Rockford, IL).

2.4. Cytochrome spectra

Spectroscopic analyses were carried out on a Beckman DU-70 spectrophotometer. Membranes that were to be analyzed spectrophotometrically were briefly sonicated and then placed in a glass cuvette with a 1-cm path length. The cuvettes were stoppered with a 7-mm sleeve-type rubber stopper if the sample was to be sparged with a gas. If anaerobic conditions were not necessary, a Teflon stopper was used. In order to record the reduced minus oxidized difference spectra, the absorbance spectrum of an air-oxidized sample was recorded and stored in memory by the spectrophotometer. The sample was then reduced with a few grains of dithionite, and the sample scanned again. The stored oxidized scan was then subtracted from the reduced scan. The procedures for CO and CN^- spectra are given in the figure legends, and closely follow procedures previously described by our lab [10,26,27]. The relative amounts of cytochromes present in membranes were calculated from $(CN^- + H_2)$ minus H_2 difference spectra using the following wavelength pairs: cytochrome *b*, 558–575 nm; cytochrome b_3, 415–430 nm; and cytochrome a_3, 444–457 nm.

2.5. O_2 uptake and cyanide inhibition assays

The oxygen consumption capacity of the membranes from the various *B. japonicum* terminal oxidase mutants was examined amperometrically, as described previously [28]. The oxygen electrode was a YSI-5331 Clark-type O_2 electrode purchased from Yellow Springs Instruments (Yellow Springs, OH). The electrode chamber (capacity = 2.4 ml) was filled with argon-sparged 0.05 M potassium phosphate buffer (pH 7.0) and aliquots of O_2-sparged buffer were added for calibration. Reduced NADH (0.5 mM) was added as a respiratory substrate to the chamber containing between 100–200 μl samples of membranes. Cyanide was injected from stock solutions to the indicated concentrations, and O_2 uptake rates were measured after 30 s. The O_2 level was maintained above 60 μM by injection of 50 μl aliquots of buffer that had been O_2-sparged. All experiments were performed at room temperature, and O_2 uptake measurements began 30 s after injection of membranes.

3. Results

Due to the functioning of multiple terminal oxidases in *B. japonicum* [29], it might be expected that a phenotypic growth disadvantage would be observed in the mutants at a specific oxygen tension or condition. However, neither significant growth rate nor cell yield differences were noted for either of the terminal

oxidase mutant strains or the double mutant compared to the wild type, when cultures were grown completely aerobically (20% partial pressure oxygen) or at 6% partial pressure O_2 in carbon-containing (MB) medium [22]. Use of other growth conditions, dependent on lower O_2 tensions, was useful to attribute a clear phenotype to the oxidase mutants (see below).

3.1. Spectral studies on membranes

Membranes from free-living aerobically grown *B. japonicum* contain cytochromes *b*, *c*, and *a* [6]: these can be readily detected by difference absorption spectral analysis of membranes. However, dithionite-reduced minus air-oxidized difference spectrum of membranes from the individual oxidase mutants were nearly identical to the spectrum from wild type cells (see Ref. [17]). For cells incubated microaerobically (1.0% O_2 condition), only a slight reduction in the amount of *b*-type cytochrome in comparison to the wild-type membranes were noted for both types of terminal oxidase mutant strains (data not shown). Our inability to see large deficiencies in (dithionite-reduced minus O_2 oxidized) spectra of membranes of the mutants is likely due to the high overall cytochrome content of *B. japonicum* membranes. Spectral studies with carbon monoxide or with cyanide as the heme-reactive ligands were useful to detect differences between the mutants and the wild-type strain. Both the wild-type strain (*JH*) and the *cox*WXYZ mutant strain (JHK12) membranes showed a large peak at 428 nm, a trough at 444 nm, and an inflection point at the 601–603 nm area; these are features indicative of a cytochrome a_3–CO complex. The wild type spectrum revealed a large peak at 415 nm, trough at 558 nm, and shoulders at 540 and 572 nm; these features are due to a cyt *b*–CO complex [30], and were clearly diminished in the *cox*WXYZ mutant strain compared to the wild type (see Ref. [17]). Compared to the wild-type membranes, the area under 415 nm peak was reproducibly diminished by 36% and the 558 nm trough by 20%, indicating the *cox*WXYZ mutant is deficient in CO-reactive cytochrome *b*.

Spectroscopic analysis of *B. japonicum* membranes from microaerobically incubated cultures of the wild type and the mutant strains were also carried out in the presence of cyanide. When we compared (CN^- plus H_2) minus O_2 difference spectra to (H_2-reduced minus O_2-oxidized) difference spectra for the wild-type membranes, we observed less (by about 20–30%) *b*-type cytochrome in the presence of cyanide than in its absence (see Ref. [17]). This result indicated the presence of CN^--reactive *b*-type cytochrome in the parent strain. In contrast, our spectral analyses on both of the terminal oxidase mutant strains (JHK12 and Bj3430) revealed that the amount of 560 nm absorbing material (i.e., heme *b*) in H_2 minus O_2 spectra was nearly the same as in (CN^- plus H_2) minus O_2 spectra. These results would be consistent with the conclusion that the mutant strains lacked some CN^- reactive cytochrome *b* component(s) that are present in the wild-type strain.

To more clearly identify the differences between the mutants and the wild type, the H_2 minus O_2 spectra were subtracted from the ($CN^- + H_2$) minus O_2 spectral scan to reveal only the cyanide reactive cytochromes. This difference absorption analysis, essentially a ($CN^- + H_2$) minus H_2 spectrum, revealed striking differences in the amount of cytochrome *b*–CN^- complex formed in the wild-type membranes versus the amount in either of the mutants. As shown in Fig. 1, for the wild-type membranes, ample CN^--reactive *b*-type cytochrome is evident from the large troughs at 558 and 428 nm, while the 444 nm trough is evidence for the presence of a cyt a_3–CN^- complex. In the *cox*WXYZ and *cox*MNOP mutant strains, the amount of CN^- reactive cytochrome *b* (note the diminished 558 and 428 nm troughs) is clearly less than in the parent strain. The *cox*WXYZ mutant also is deficient in the amount of cytochrome a_3 (diminished trough at 444 nm compared to the wild type). From several experiments like those shown, strains JHK12 (*cox*WXYZ mutant) and Bj3430 (*cox*MNOP mutant) retain less than 32% and 40%, respectively, of the wild type amount of cytochrome b_3 based on the trough size at 428 nm, and about 55% and 80%, respectively, of the wild type level of CN^--reactive cytochrome *b* (based on the trough size at 558 nm). In CN^- difference spectra, membranes from the double mutant strain (scan D in Fig. 1) lacking both *cox*WXYZ and *cox*MNOP, was only slightly deficient in cytochrome a_3, but was severely deficient in cytochrome b_3 (see 428 nm area) and in total cytochrome *b* (see 558 nm): it retained less than

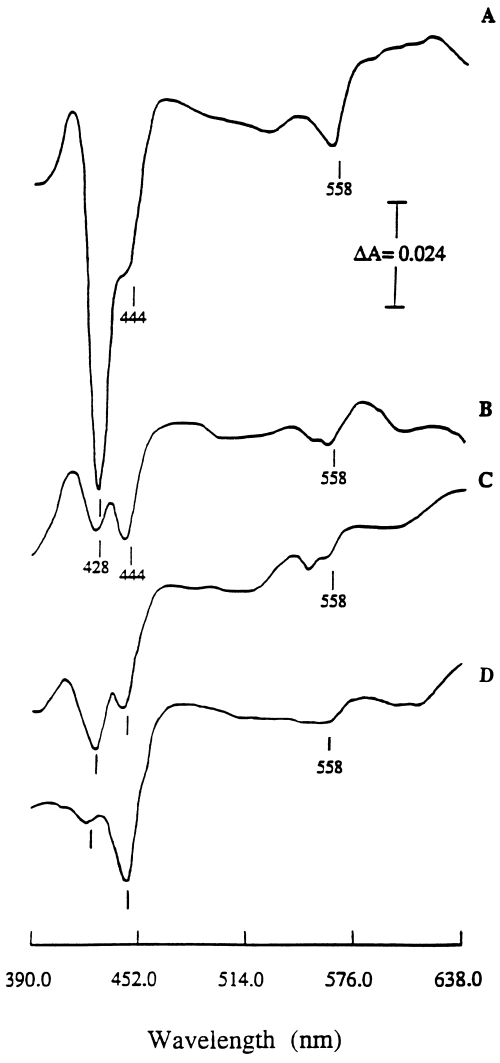

Fig. 1. $(CN^- + H_2)$ minus H_2 spectra of *B. japonicum* membranes from strains JH (A; wild type), JHK12 (B; *cox*X Km^r insertion mutant), Bj3430 (C; Δ*cox*N mutant) and KS4 (*cox*X *cox*N double mutant). The H_2 minus O_2 spectrum was recorded as background and subtracted from the $(CN^- + H_2)$ minus O_2 spectrum resulting in a $(CN^- + H_2)$ minus H_2 spectrum. The membrane protein concentration of strains JH, JHK12 and JHKS4 were all 3.0 mg ml^{-1}; and for Bj3430, 1.4 mg ml^{-1}. Scans B and C for the mutants are directly comparable to the weight scan shown (scan A), but scan D was done at a different time; thus for calculation purposes, it was compared to a separate wild type scan (not shown) done on a membrane preparation made along with and scanned the same time as scan D.

7% of the cytochrome b_3 and about 25% of the total CN^- reactive cytochrome *b* of the parent. This result, along with the cyanide titration curves (see below), are consistent with the interpretation that the double mutant indeed lacks both of the oxidase components that are lacking in each of the individual *cox* mutant strains.

3.2. Oxygen uptake by membranes

There was no noticeable difference in NADH dependent respiration rates by membrane particles of the individual oxidase mutants (strains JHK12 and Bj3430) compared to the wild type at all O_2 concentrations tested, from 50 to 250 μM O_2. However, membranes of the double mutant (strain JHKS4) consistently took up O_2 at rates of 52–60% of the wild type in O_2 levels below 100 μM (30–100 μM). These rate differences were seen for membranes from cells that had been subjected to the microaerobic incubation condition, and in a typical assay were about 200 nmol O_2 consumed per minute per milligram protein for the wild type and 115 such units for the double mutant. Similarly, the H_2-dependent respiration rates of membranes for the double mutant were about one-half that of the wild type (data not shown) when assayed at O_2 levels below 120 μM.

3.3. Cyanide inhibition of oxidase activity

As an inhibitor of terminal oxidase activity, cyanide is useful to determine the number of CN^--reactive (O_2-binding) components in complex branched respiratory chains. We titrated the oxidase activity as a function of different cyanide concentrations ranging from 2×10^{-8} to 5×10^{-4} M to assess the complement of terminal respiratory components in membranes from microaerobically incubated cells of the wild type (Fig. 2A), and for strains JHK12 (Fig. 2B) and Bj3430 (Fig. 2C). The inhibition of O_2 uptake by CN^- for wild type membranes was triphasic, with K_i's of approximately 0.1, 0.7, and at about 50 μM inhibitor. Both of the mutant strains contained the component with the highest affinity for CN^-, but they each exhibited a clearly different inhibition pattern than was seen for the wild type in the 1.0 μM cyanide concentration area. Specifically, both mutant strains exhibited a smaller decrease in activity than the wild type strain, especially in the 0.3 to 1.5 μM range of inhibitor. The net result is that the membranes from the mutants were less subject to respira-

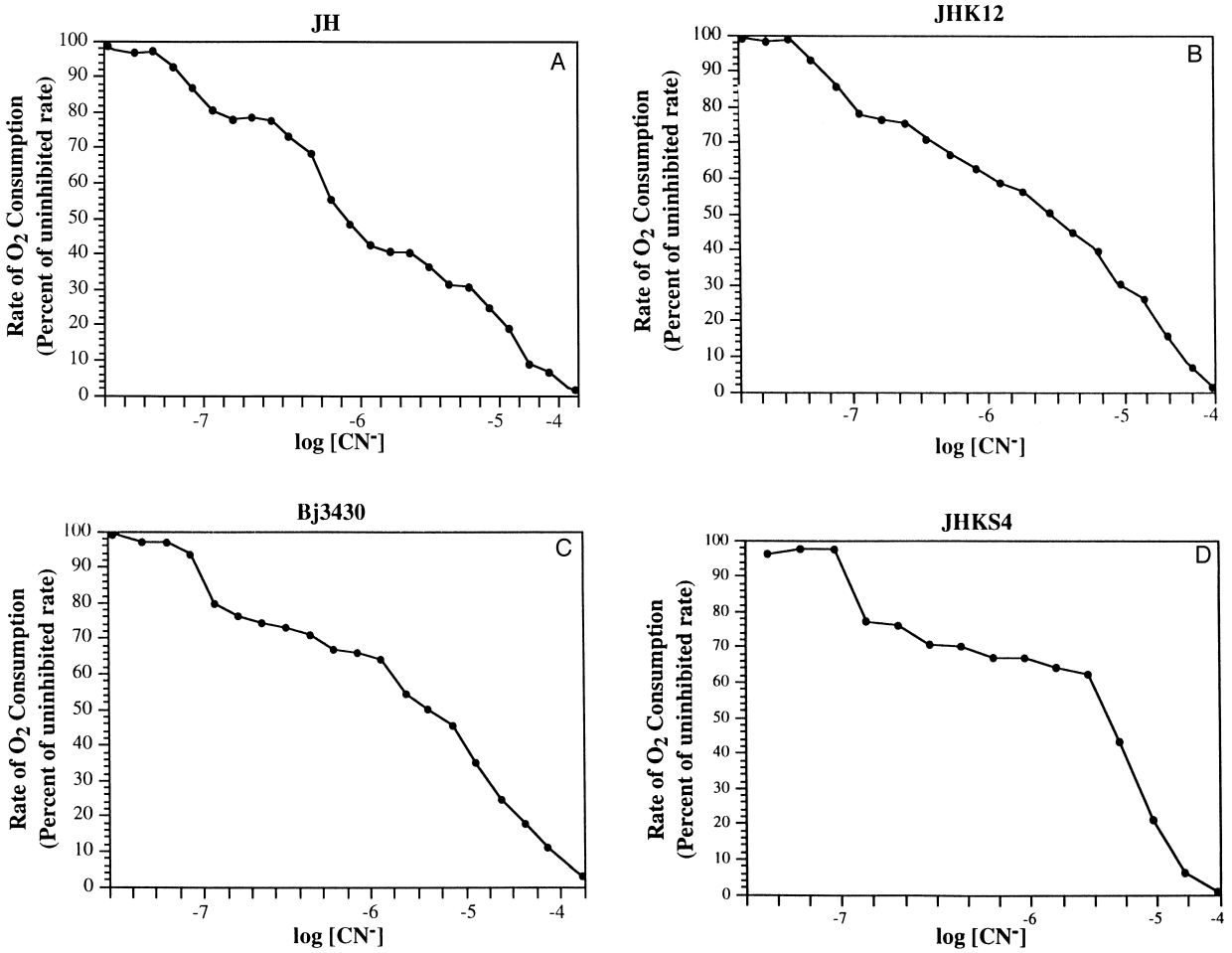

Fig. 2. Cyanide titration curves of membranes from free-living cells that had been incubated microaerobically for three days. The rate of O_2 uptake was measured amperometrically in oxygen concentrations of 60–120 μM. Aqueous solutions of cyanide were injected into a 2.4-ml capacity electrode chamber that contained actively respiring membranes suspended in 0.05 M potassium phosphate buffer (pH 7.0), and containing reduced NADH (0.5 mM) as reductant. The rates of membrane O_2 uptake activities (nmol O_2 consumed per minute per milligram protein) in 100–120 μM O_2 without inhibitor were: strain JH (229), strain JHK12 (208); strain Bj3430 (211), strain JHKS4 (132). The membrane protein concentrations (mg/ml) were: strain JH (3.3); JHK12 (2.8); Bj3430 (3.2) JHKS4 (3.8) and 100–250 μl aliquots of these membranes were injected into the chamber prior to CN^- injection.

tory inhibition in most areas of the CN^- titration curve below 2.0 μM inhibitor. For example, from six separate CN^- titration experiments on membranes isolated from independent cultures we found that 2.0 μM CN^- inhibited approximately 60% of the total oxidase activity of the wild type, but 40% of the total O_2 uptake activity of *cox*WXYZ mutant strain and 38% of the O_2 uptake activity of *cox*MNOP mutant strain membranes.

Although similar to each other, the membranes isolated from *cox*WXYZ and *cox*MNOP mutant strains were not identical in their inhibition patterns; they had subtle but reproducible differences in their CN^- inhibition curves. *cox*MNOP mutant membranes were reproducibly more resistant to CN^- in the 0.3 to 1.0 μM cyanide inhibition area. Like the wild type, both mutant strains contained an inhibition phase in the 5–50 μM cyanide inhibition area. This latter inhibition phase was clearly attributable to cytochrome aa_3, as strain LO501 (Δ*cox*A) lacked this inhibitory phase; the inhibition of respiration was nearly complete in strain LO501 membranes at 1.0 μM CN^- [17]. The CN^- inhibition data indicate that at least four terminal oxidases are present in mi-

croaerobically incubated cells, and we can conclude that the two 'medium' affinity components are encoded by *cox*WXYZ and *cox*MNOP. Possible candidates for the component inhibited in the 0.1 μM inhibition phase are the FixNOQP complex [7], the putative flavoprotein oxidase [26], or the non-heme respiratory component [31]. All of these components are inhibited by cyanide.

Membranes from the *cox*WXYZ *cox*MNOP double mutant strain (JHKS4) had a broad area of cyanide resistance to the inhibitor concentrations from 0.10 μM to 5.0 μM (Fig. 2D). The results for the double mutant are consistent with the interpretation that this strain lacks both of the 'medium affinity' components, but retains both the most and the least cyanide sensitive factors. Performance of these cyanide titration inhibition experiments on membranes from cells grown aerobically (20% partial pressure O_2) revealed a different sensitivity pattern to CN^- than for the microaerobically incubated cells. The bulk of the inhibition (greater than 75%) for all the strains when grown in high aeration was at concentrations above 5.0 μM CN^- (data not shown). It is well established that the primary terminal oxidase in this strain of *B. japonicum* grown in high-aeration conditions is cytochrome aa_3 [18].

3.4. Chemolithotrophic growth

The terminal oxidases described here are expressed after incubation of cells in a low O_2 environment. Therefore, we tested the growth abilities of the strains under conditions of H_2-dependent chemolithotrophic growth, which requires low levels of O_2. As shown in Fig. 3, the wild type doubled in five days, as did the individual oxidase mutants (strains JHK12 and Bj3430), but the double mutant grew more slowly chemolithotrophically, taking 16 days to double. The results indicate the importance of these oxidases for low O_2 growth, and that either one of the two oxidases will suffice to permit the chemolithotrophic metabolism. Whole cell H_2 uptake assays from 14 day chemolithotrophically grown cells (assayed in the presence of 100–120 μM oxygen) revealed that the double mutant had H_2 oxidation rates 31% of the wild type; the rates were (nmol/h per 10^8 cells, mean $\pm$ standard deviation) 1664 ± 112 for the wild type and 520 ± 42 for strain JHKS4. The lower levels by the (double) mutant undoubtedly reflect a deficiency in O_2-dependent respiration, rather than a deficiency in hydrogenase per se.

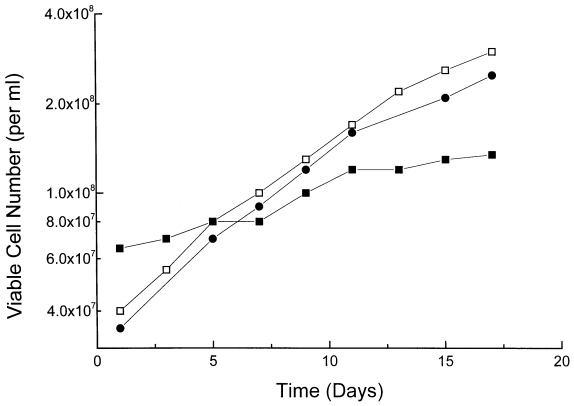

Fig. 3. H_2-dependent chemolithotrophic growth of strains JH (□), JHK12 (●), and JHKS4 (■) strains upon inoculation into a mineral salts medium (see text) and incubation in an atmosphere of 10% H_2, 5% CO_2, 1% O_2 (balance N_2), and incubated at 30°C. Samples were removed every two days over a 17-day period for viable cell number determinations based on standard curves of OD_{540} measurements vs. viable cell number. The standard curve was determined for cells from that same growth condition. Strain Bj3430 (not shown) had a growth curve similar to strains JH and JHK12.

4. Discussion

Because of the agricultural and economic importance of soybean crops, it is of interest to understand the factors that contribute to the optimal functioning of *B. japonicum*, both as a free-living organism and as a microsymbiont of soybean. The focus on terminal oxidases as one of these factors is justified, considering that the bacterium must supply ATP, via respiration-driven mechanisms, to fuel nitrogenase directly as well as for many of the ancillary bacterial processes required for efficient symbiotic N_2 fixation. The roles of already sequenced genes encoding terminal oxidase complexes can be addressed in a straightforward manner by studying gene-specific mutants in the oxidases or the levels of mRNA corresponding to that gene. The roles of two *B. japonicum* membrane-bound complexes within the heme–copper family of terminal oxidases, *Fix*NOQP

and *Cox*BA, have been addressed through a combination of mutant analyses [12–14], purification of the complex [7,10], or quantification of both spectral and transcript signals [18,19]. All of these studies are consistent with the *Cox*BA playing a respiratory role in high-aeration conditions (i.e., in free-living culture) and *Fix*NOQP playing a role in a very low O_2 environment (i.e., in symbiosis).

*Cox*MNOP and *Cox*WXYZ do not play such clearly defined roles, but from our studies, it can be concluded they must be important in some aspect of microaerobic metabolism. In a strain lacking both of these oxidases, low O_2 growth (H_2-dependent chemolithotrophy) is severely affected. For chemolithotrophic growth, the double mutant is likely severely energy-deprived, as these cultures are dependent on H_2/O_2 respiration at O_2 levels below 12.5 μM, and it was shown this strain is H_2 oxidation-deficient (with O_2 as terminal acceptor) in O_2 levels at and below 120 μM. The results are consistent with the conclusion that these oxidases function microaerobically. Other bacteria capable of H_2-dependent chemolithotrophic growth, such as *Rhodobacter*, *Rhodospirillum*, and *Paracoccus* (see Ref. [32]) also contain terminal oxidases in the cyt *c* oxidase and ubiquinol oxidase groups [11], but the roles of these in microaerobic or chemolithotrophic metabolism are not known.

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