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Enzymatic Characterization and *In Vivo* Function of Five Terminal Oxidases in *Pseudomonas aeruginosa*

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The ubiquitous opportunistic pathogen *Pseudomonas aeruginosa* has five aerobic terminal oxidases: bo_3-type quinol oxidase (Cyo), cyanide-insensitive oxidase (CIO), aa_3-type cytochrome *c* oxidase (aa_3), and two cbb_3-type cytochrome *c* oxidases (cbb_3-1 and cbb_3-2). These terminal oxidases are differentially regulated under various growth conditions and are thought to contribute to the survival of this microorganism in a wide variety of environmental niches. Here, we constructed multiple mutant strains of *P. aeruginosa* that express only one aerobic terminal oxidase to investigate the enzymatic characteristics and *in vivo* function of each enzyme. The K_m values of Cyo, CIO, and aa_3 for oxygen were similar and were 1 order of magnitude higher than those of cbb_3-1 and cbb_3-2, indicating that Cyo, CIO, and aa_3 are low-affinity enzymes and that cbb_3-1 and cbb_3-2 are high-affinity enzymes. Although cbb_3-1 and cbb_3-2 exhibited different expression patterns in response to oxygen concentration, they had similar K_m values for oxygen. Both cbb_3-1 and cbb_3-2 utilized cytochrome c_4 as the main electron donor under normal growth conditions. The electron transport chains terminated by cbb_3-1 and cbb_3-2 generate a proton gradient across the cell membrane with similar efficiencies. The electron transport chain of aa_3 had the highest proton translocation efficiency, whereas that of CIO had the lowest efficiency. The enzymatic properties of the terminal oxidases reported here are partially in agreement with their regulatory patterns and may explain the environmental adaptability and versatility of *P. aeruginosa*.

Pseudomonas aeruginosa is an opportunistic pathogen responsible for severe nosocomial infections in immunocompromised individuals and chronic lung infections in patients with the genetic disease cystic fibrosis. The bacterium is ubiquitously distributed in soil and water environments and infects not only humans but also a wide variety of animals, insects, and plants (1). The ubiquity of *P. aeruginosa* is partly attributed to its ability to utilize various carbon sources and generate energy through multiple pathways (2–5). *P. aeruginosa* grows by aerobic respiration in the presence of oxygen or by dissimilatory nitrate respiration (denitrification) and arginine fermentation under anaerobic conditions (6, 7).

The genome of *P. aeruginosa* encodes at least five aerobic terminal oxidases, bo_3-type oxidase (Cyo), aa_3-type oxidase (aa_3), cyanide-insensitive quinol oxidase (CIO), and two isoforms of cbb_3-type oxidase (cbb_3-1 and cbb_3-2), which catalyze the four-electron reduction of molecular oxygen to water as the final reaction of the aerobic respiratory chain (5, 8). Cyo is encoded by the *cyoABCDE* genes (PA1317 to -1321), which are highly homologous with the corresponding genes of *Escherichia coli*, while aa_3 is encoded by the *coxBA-coxC* (*coIII*) genes (PA0105 to -0108). The *cioAB* genes (PA3930-PA3929) encode CIO, which has homology to *bd*-type quinol oxidases (9), while the *ccoN1O1Q1P1* (*cco1* gene cluster; PA1554 to -1552) and *ccoN2O2Q2P2* genes (*cco2* gene cluster; PA1557 to -1555) encode cbb_3-1 and cbb_3-2, respectively. The known features of the five terminal oxidases of *P. aeruginosa* are summarized in Table S1 in the supplemental material.

Two groups of terminal oxidases are associated with aerobic respiration: the cytochrome *bd* family and the heme-copper oxidoreductase superfamily. CIO belongs to the cytochrome *bd* family, and although its primary structure is similar to those of canonical *bd* oxidases, it is phylogenetically distinct (9–11). Recent analyses in *Gluconobacter oxydans* showed that the CIO contains hemes b_{558}, b_{595}, and *d*, as in the case of canonical *bd* oxidases, and has a high turnover rate (12, 13). The cytochrome *bd* oxidase of *E. coli* has high affinity for oxygen, whereas CIOs of *Campylobacter jejuni* and *G. oxydans* have low affinity for oxygen (13, 14). In contrast, the CIO of *P. aeruginosa* was predicted to have high affinity for oxygen because a *cco1 cco2* double mutant, which lacked cbb_3-1 and cbb_3-2, was able to grow under microaerobic conditions (2% O_2), but a *cco1 cco2 cio* triple mutant, which lacked cbb_3-1, cbb_3-2, and CIO, did not grow under such conditions (15). However, the affinity of *P. aeruginosa* CIO for oxygen has not been determined to date.

The expression of CIO in *P. aeruginosa* is induced in the stationary phase or under low-oxygen or copper limitation conditions. Inactivation of the other terminal oxidases by respiratory inhibitors or gene disruption also leads to significant upregulation of the *cio* genes (15–21). The upregulation of CIO in the stationary phase is probably because *P. aeruginosa* produces cyanide, which inhibits heme-copper oxidases, in the stationary phase. The *cio* operon is positively regulated by RoxSR, which is a two-component regulatory system that may sense respiratory chain electron flow or redox status of the quinone pool, and is negatively regulated by ANR (anaerobic regulator of arginine deiminase and nitrate reductase), which is a low-oxygen-responsive global regulator (17, 18). The stationary-phase sigma factor RpoS also activates expression of the *cio* genes (21).

The terminal oxidases Cyo, aa_3, cbb_3-1, and cbb_3-2 belong to the heme-copper oxidoreductase superfamily, whose members

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have a conserved catalytic core subunit, which contains a low-spin heme and a binuclear catalytic center, consisting of a high-spin heme and copper atom (Cu_B). Enzymes of this family are classified into three main types (A, B, and C) based on features of the core subunit (22). Cyo and aa_3 of *P. aeruginosa* are type A enzymes, whereas cbb_3-1 and cbb_3-2 are type C enzymes. Cyo does not carry a Cu_A center, which receives electrons from cytochrome *c*, and is therefore considered to be a quinol oxidase. The *cyo* genes are expressed at low levels under normal growth conditions in LB medium but are upregulated by iron starvation (21). aa_3 is closely related to mitochondrial terminal oxidases. aa_3-type oxidases typically have low affinity for oxygen and function as main enzymes under high-oxygen conditions in many bacterial species (23–26). However, in *P. aeruginosa*, aa_3 is expressed at very low levels in nutrient-rich medium under high-oxygen conditions and is upregulated only in response to nutrient starvation (21).

cbb_3-1 and cbb_3-2 are exclusively found in bacteria and are the most phylogenetically distant members of the heme-copper oxidoreductase superfamily (27). cbb_3-type oxidases have very high affinity for oxygen. For example, the reported K_m values for cbb_3 oxidases of *Bradyrhizobium japonicum* and *Campylobacter jejuni* are 7 and 40 nM, respectively (14, 28). cbb_3-type oxidases are induced under low-oxygen conditions in many bacteria or are the only terminal oxidase in several obligately microaerophilic bacteria, indicating that cbb_3 oxidases are the major terminal oxidases in low-oxygen environments (14, 29–32). Similar to cbb_3 oxidases from other bacteria, cbb_3-2 of *P. aeruginosa* is highly induced under low-oxygen conditions. cbb_3-2 is also induced in the stationary phase under aerobic conditions, probably because of low dissolved oxygen concentration due to high cell density. In contrast, cbb_3-1 is constitutively expressed and is the major oxidase that functions in the exponential phase under high-oxygen conditions (15, 17). Thus, *P. aeruginosa* is unique in that it expresses cbb_3-type oxidases as the dominant respiratory enzymes, even under high-oxygen conditions, under normal laboratory growth conditions.

In *P. aeruginosa*, the five known terminal oxidases are differentially regulated in response to growth conditions. ANR, RoxSR, the iron-responsive regulator Fur, and the stationary-phase sigma factor RpoS are involved in the regulation of these enzymes in response to oxygen concentration, presence of respiratory inhibitors, iron availability, and nutrient conditions (see Table S1 in the supplemental material) (5, 21). The presence of multiple terminal oxidases with distinct enzymatic features and the ability to regulate the expression of the enzymes that are most suitable for the growth conditions likely contribute to the ubiquity of *P. aeruginosa* in the environment. To better understand the mechanisms that allow *P. aeruginosa* to survive in changing environments, detailed analyses of the enzymatic features and *in vivo* function of each terminal oxidase are necessary. However, because multiple terminal oxidases are simultaneously expressed in *P. aeruginosa* cells, *in vivo* functional characterization of individual enzymes is difficult. In this work, we constructed multiple mutant strains of *P. aeruginosa* that express only one terminal oxidase to characterize the features of each enzyme.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *P. aeruginosa* PAO1ut, which was used as a wild-type strain in this work, is a PAO1 strain that is maintained in our laboratory (21). Cells were routinely grown in LB medium at 37°C. For aerobic cultivation, Erlenmeyer flasks or test tubes with gas-permeable plugs were used. For microaerobic cultivation, square bottles were used and the medium was continuously bubbled through a sparger with a gas mixture consisting of 2% O_2 and 98% N_2. For anaerobic cultivation, test tubes sealed with butyl rubber stoppers were used, the medium was supplemented with 100 mM sodium nitrate, and the gas phase was replaced with argon. For aerobic cultivation of the derivatives of *E. coli* MB43, square bottles were used and LB medium was continuously bubbled with air through a sparger. Bacterial colonies were isolated on LB agar plates or *Pseudomonas* isolation agar (BD Difco Laboratories, Sparks, MD). The AnaeroPack System (Mitsubishi Gas Chemical, Tokyo, Japan) was used for the anaerobic incubation of plates. The concentrations of the antibiotics were as follows: 100 μg/ml ampicillin and 12.5 μg/ml tetracycline (Tc) (for *E. coli*) and 200 μg/ml carbenicillin (Cb) and 150 μg/ml Tc (for *P. aeruginosa*). When necessary, 5% (wt/vol) sucrose or 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium.

DNA manipulations. Recombinant DNA experiments were performed using standard methods (33). DNA was introduced into *P. aeruginosa* strains by transconjugation with *E. coli* strain S17-1 or by electroporation. *Ex Taq* (TaKaRa, Kyoto, Japan) and KOD Plus polymerases (Toyobo, Osaka, Japan) were used for PCRs. Synthetic oligonucleotides were obtained from Sigma-Genosys (Hokkaido, Japan) or Greiner Bio One (Tokyo, Japan). The primers used for PCR are listed in Table S2 in the supplemental material.

Construction of mutant strains. The *cyoABCDE*, *ccoN1O1Q1P1*, and *ccoN2O2Q2P2* gene clusters were knocked out using the Flp-*FRT* recombination system (34) with constructs pEX-Δcyo, pEX-Δcco1, and pEX-Δcco2, respectively, according to the method described previously (35). For construction of pEX-Δcyo, 0.9- and 1.4-kb fragments containing the upstream and downstream flanking regions of the *cyoABCDE* gene cluster, respectively, were amplified by PCR from PAO1ut chromosomal DNA using primer sets BoR1/BoR2 and BoR3/BoR4, respectively, and were then tandemly inserted into the EcoRI-BamHI and BamHI-PstI sites, respectively, of pEX18Ap (34). The resulting construct was digested with BamHI, blunt ended by the Klenow reaction, and then ligated with a blunt-ended SacI-excised fragment containing the *tet-FRT* cassette from pPS854*tet* (35), resulting in pEX-Δcyo. pEX-Δcco1 and pEX-Δcco2 were constructed in the same manner using primer sets cbR3/cbR4 and cbR5/cbR6 and primer sets cbR1/cbR2 and cbR3/cbR4, respectively.

The *cioAB* and *coxBA*-PA0107-*coxC* gene clusters were knocked out by in-frame deletion with plasmids pEX-Δcio and pEX-Δcox, respectively, according to the method described previously (21). These plasmids were constructed using the same method as that for pEX-Δcyo, except that the *tet-FRT* cassette was not inserted. The primer sets used for construction of pEX-Δcio and pEX-Δcox were ciR1/ciR2 and ciR3/ciR4 and sets aaR1/aaR2 and aaR3/aaR4, respectively.

Mutant strains lacking the *cc4*, *cycB*, PA4571, or PA5491 gene were constructed by in-frame deletion using the same method as that for the *cio* and *cox* mutants with plasmids pEX-Δcc4, pEX-ΔcycB, pEX-ΔPA4571, and pEX-ΔPA5491, respectively. The primer sets used to amplify the upstream and downstream flanking regions of the respective genes for the construction of pEX-Δcc4, pEX-ΔcycB, and pEX-ΔPA4571 were cc4-d/cc4-c and cc4-b/cc4-a, cycB-d/cycB-c and cycB-b/cycB-a, and PA4571-a/PA4571-b and PA4571-c/PA4571-d, respectively. For construction of pEX-ΔPA5491, 1.4-kbp PCR fragments containing the upstream and downstream flanking regions of PA5491 were amplified from PAO1ut chromosomal DNA using primer sets PA5491-d/PA5491-c and PA5491-b/PA5491-a, respectively. The amplified fragments were used as the templates for a second PCR using the primer set PA5491-d/PA5491-a. The amplified fragment, which carried the upstream and downstream flanking regions of PA5491 but lacked the PA5491 coding region, was inserted into the XbaI-HindIII sites of pEX18Ap, resulting in pEX-ΔPA5491.

Strains with multiple mutations were constructed by stepwise mutation of the terminal oxidase genes. Because strains QXAa and PTO5, which lacked the *cyo*, *cio*, *cco1*, and *cco2* gene clusters, could not be ob-

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
P. aeruginosa		
PAO1ut	Our laboratory strain of PAO1 used as a wild type	21
QXBo	Deletion of *cioAB*, *coxBA-coxC*, *ccoN1O1Q1P1*, and *ccoN2O2Q2P2*	This study
QXCi	Deletion of *cyoABCDE*, *coxBA-coxC*, *ccoN1O1Q1P1*, and *ccoN2O2Q2P2*	This study
QXAa	Deletion of *cyoABCDE*, *cioAB*, *ccoN1O1Q1P1*, and *ccoN2O2Q2P2*	This study
QXAaS2	Suppressor mutant of QXAaS2; able to grow aerobically	This study
QXCb1	Deletion of *cyoABCDE*, *cioAB*, *coxBA-coxC*, and *ccoN2O2Q2P2*	This study
QXCb2	Deletion of *cyoABCDE*, *cioAB*, *coxBA-coxC*, and *ccoN1O1Q1P1*	This study
PTO5	Deletion of *cyoABCDE*, *cioAB*, *coxBA-coxC*, *ccoN1O1Q1P1*, and *ccoN2O2Q2P2*	This study
QXCb1Δ*cc4*	*cc4* mutant of QXCb1	This study
QXCb1Δ*cycB*	*cycB* mutant of QXCb1	This study
QXCb1ΔPA4571	PA4571 mutant of QXCb1	This study
QXCb1ΔPA5491	PA5491 mutant of QXCb1	This study
QXCb2Δ*cc4*	*cc4* mutant of QXCb2	This study
QXCb2Δ*cycB*	*cycB* mutant of QXCb2	This study
QXCb2ΔPA4571	PA4571 mutant of QXCb2	This study
QXCb2ΔPA5491	PA5491 mutant of QXCb2	This study
E. coli		
JM109	Host strain for DNA manipulation	33
S17-1	C600::RP-4 2-(Tc::Mu)(Kan::Tn*7*) *thi pro hsdR hsdM*$^+$ *recA*	55
MB43	*cydB cyoB appB nuoB*, nonmarker derivative of MB44	43
Plasmids		
pUC18	Cloning vector; Ap^r	33
pEX18Ap	Gene replacement vector; Ap^r Cb^r *oriT*$^+$ *sacB*$^+$	34
pFLP2	Flp recombinase-expressing plasmid; Ap^r	34
pMMB67EH	IncQ, expression vector; Ap^r Cb^r	56
pPS854*tet*	*tet-FRT* cassette, Ap^r Tc^r	35
pEX-Δcyo	Plasmid for *cyoABCDE* mutation, a derivative of pEX18Ap, Tc^r	This study
pEX-Δcio	Plasmid for *cioAB* mutation, a derivative of pEX18Ap	This study
pEX-Δcox	Plasmid for *coxBA-coxC* mutation, a derivative of pEX18Ap	This study
pEX-Δcco1	Plasmid for *ccoN1O1Q1P1* mutation, a derivative of pEX18Ap, Tc^r	This study
pEX-Δcco2	Plasmid for *ccoN2O2Q2P2* mutation, a derivative of pEX18Ap, Tc^r	This study
pEX-Δcc4	Plasmid for *cc4* mutation, a derivative of pEX18Ap	This study
pEX-ΔcycB	Plasmid for *cycB* mutation, a derivative of pEX18Ap	This study
pEX-ΔPA4571	Plasmid for PA4571 mutation, a derivative of pEX18Ap	This study
pEX-ΔPA5491	Plasmid for PA5491 mutation, a derivative of pEX18Ap	This study
pMMB-*cc4*	*cc4* on pMMB67EH	This study
pMMB-PA5491	PA5491 on pMMB67EH	This study
pMMB-*cioAB*	*cioAB* on pMMB67EH	This study
pUC-*cioAB*	*cioAB* on pUC18	This study
pUC-*cydAB*	*cydAB* of *E. coli* on pUC18	This study

[a] Ap^r, ampicillin resistant; Cb^r, carbenicillin resistant; Tc^r, tetracycline resistant.

tained by aerobic cultivation, they were isolated under anaerobic cultivation conditions.

Construction of complementation plasmids. pMMB-*cc4* and pMMB-PA5491 were constructed by insertion of PCR fragments carrying the *cc4* and PA5491 genes, which were amplified with primer sets cc4-F/cc4-R and PA5491-F/PA5491-R, respectively, into the BamHI-HindIII and SacI-BamHI sites of pMMB67EH, respectively. pUC-*cioAB* was constructed by insertion of a PCR fragment carrying the *cioAB* genes, which was amplified with primers cio1 and cio2 from PAO1ut chromosomal DNA, into the EcoRI-XbaI sites of pUC18. pMMB-*cioAB* was constructed by insertion of the same fragment into pMMB67EH. pUC-*cydAB* was constructed by insertion of a PCR fragment carrying the *cydAB* genes, which was amplified with primers cyd1 and cyd2 from *E. coli* JM109 chromosomal DNA, into the BamHI-HindIII sites of pUC18.

Measurement of oxygen consumption. Preparation of membrane fractions and measurements of oxygen consumption were performed according to the methods described by Cunningham and Williams (10). Briefly, *P. aeruginosa* strains were grown in LB medium until the optical density at 600 nm (OD_{600}) of the culture reached approximately 0.85. Cells were pelleted by centrifugation at 6,000 × *g*, washed twice with 50 mM potassium phosphate buffer (pH 7.0), and resuspended in 10 mM potassium phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$. DNase at 0.01 mg/ml (final concentration) was added to the cell suspension, and the cells were then disrupted by passing the suspension twice through a French pressure cell at 16,000 lb/in^2. Unbroken cells were removed by centrifugation at 6,000 × *g*. The membrane fraction was collected by centrifugation at 100,000 × *g* for 1 h at 4°C and resuspended in 10 mM potassium phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$ to give a protein concentration between 10 and 20 mg/ml. The protein concentration was determined by the Bradford method using a protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

Oxygen consumption activity was measured amperometrically using

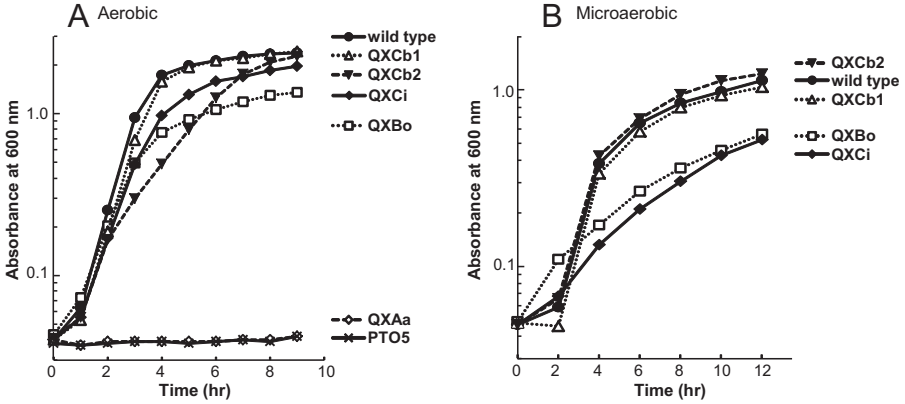

FIG 1 Growth profiles of the quadruple terminal oxidase mutant strains of *P. aeruginosa*. (A) Growth under aerobic conditions. The strains were cultivated in 40 ml LB medium in 300-ml Erlenmeyer flasks with shaking at 180 rpm. (B) Growth under microaerobic conditions (2% O_2). The strains were cultivated in 150 ml LB medium in 300-ml square bottles. The medium was bubbled with a gas mixture consisting of 2% O_2 and 98% N_2 at a gas flux rate of 150 ml/min. The data shown are representatives from at least three independent experiments. The same plots in linear scale are shown in Fig. S4 in the supplemental material.

an Apollo 4000 free radical analyzer equipped with a 2-mm Iso-Oxy-2 O_2 electrode (WPI, Sarasota, FL). The reaction was performed in a multiport chamber (WPI) at 25°C. The reaction mixture (2.5 ml) contained 33 mM potassium phosphate (pH 7.0) and 0.5 mM NADH or 0.1 mM tetramethylene phenylene diamine (TMPD) and 5 mM ascorbate. When necessary, 1 mM KCN or 10 μg/ml antimycin A was added to the reaction mixture. The reaction was started by adding a volume of the membrane fraction equivalent to 0.5 mg protein to the air-saturated reaction mixture (O_2 concentration was assumed to be 240 μM at 25°C), and the oxygen consumption activity was calculated by the decline of the O_2 concentration.

Determination of oxygen affinity. For determination of the K_m values for oxygen using an oxygen electrode, membrane fractions from each of the terminal oxidase mutants were prepared as described above. The reaction was performed in an anaerobic vial (68-ml total volume) equipped with an Iso-Oxy-2 O_2 electrode (WPI), and the reaction mixture (64 ml) contained 33 mM potassium phosphate (pH 7.0) and 30 mM malate. After most of the oxygen in the vial was removed by bubbling argon gas for 10 min, a volume of the membrane fraction equivalent to 0.5 mg protein was added to the reaction buffer. The consumption of dissolved oxygen was monitored with an Apollo 4000 free radical analyzer (WPI) and the K_m values for oxygen were determined from Hanes-Woolf plots (see Fig. S1 in the supplemental material).

Determination of the K_m values using deoxygenation kinetics of oxymyoglobin or oxyleghemoglobin was performed according to the method described previously (36–38). Membrane fractions were prepared as described above except that 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA was used for washing and resuspension of the cells. Equine heart myoglobin (Sigma-Aldrich, St. Louis, MO) and partially purified leghemoglobin from the nodules of pea (*Pisum sativum*), which was kindly provided by N. Suganuma (Aichi University of Education), were reduced with dithionite and desalted by passage through a PD-10 column (GE Healthcare). The concentrations of myoglobin and leghemoglobin were determined by the spectrum of alkaline pyridine hemochrome using an extinction coefficient value for pyridine hemochrome of 33.9 mM^{-1} cm^{-1} at 556 nm (39). The reaction was performed in a sealed cuvette containing 5 ml of the degassed reaction mixture, which consisted of 20 mM potassium phosphate (pH 7.0), 1 mM EDTA, 15 mM D,L-malate, and 19.3 mM oxymyoglobin or oxyleghemoglobin. After addition of the membrane fraction sample (20 to 70 μl), the deoxygenation of oxymyoglobin or oxyleghemoglobin was continuously monitored at 581 and 563 nm or 572 and 560 nm, respectively, using a U-3210 or U-2910 spectrophotometer (Hitachi, Tokyo, Japan). The concentration of oxygen in the reaction mixture and the oxygen consumption rate at several time points were calculated according to the method described by Bergersen and Turner (36). The values for the dissociation constant used in the calculations were 0.786 and 0.05 μM for oxymyoglobin and oxyleghemoglobin, respectively. The K_m values for oxygen were determined from Eadie-Hofstee plots (see Fig. S2 and S3 in the supplemental material).

Determination of proton translocation efficiency. *P. aeruginosa* strains were grown under aerobic conditions in LB medium until the OD_{600} reached 1.3 to 1.6. *E. coli* MB43 derivatives used for determination of the H^+/O ratio of CIO and the *bd* oxidase 1 were grown for 24 h. All cells were collected by centrifugation at 10,200 × *g* for 10 min at 4°C and washed twice with wash buffer, consisting of 300 mM sucrose and 10 mM $MgCl_2$. The cell pellet was resuspended in wash buffer at an OD_{600} of 200 to 300. The H^+/O ratio of the cell suspension was determined by measuring the changes of pH upon oxygen pulse using a pH electrode (6066-10C; Horiba, Kyoto, Japan) connected to a pH meter (F-53; Horiba). The oxygen pulse reaction was performed at 25°C in an anaerobic vessel equipped with the pH electrode. The reaction mixture contained 0.5 mM morpholinepropanesulfonic acid (MOPS) (pH 7.0), 100 mM KCl, 100 mM KSCN, 30 mM sucrose, 1 mM $MgCl_2$, and 800 μl of the cell suspension in a total volume of 8 ml. Electron donors were not added to the reaction mixture because the cells had measurable oxygen consumption activity using endogenous electron donors and the addition of exogenous substrates had no effect on the activity, as was reported for *Rhodobacter* species (40). After the mixture was flushed with argon and the pH was adjusted to 7.0 with 1 N KOH, the reaction was started by adding a defined amount of oxygen with 60 μl of air-equilibrated water. Calibration of the pH electrode was done by the addition of 50 μl of deoxygenated 3 mM HCl. The H^+/O ratio was calculated from the pH change and the amount of added oxygen.

RESULTS

Growth profile of multiple terminal oxidase knockout mutants. Five terminal oxidase quadruple mutant strains, QXBo, QXCi, QXAa, QXCb1, and QXCb2, which carried only one complete terminal oxidase gene cluster, encoding Cyo, CIO, aa_3, cbb_3-1, and cbb_3-2, respectively, and a quintuple mutant strain, PTO5, which lacked all five terminal oxidase gene clusters, were constructed by repeated homologous recombination (Table 1). Strains QXBo, QXCi, QXCb1, and QXCb2 were able to grow aerobically, whereas strains QXAa and PTO5 did not grow under this condition (Fig. 1A; also see Fig. S4A in the supplemental material). These results indicated that Cyo, CIO, cbb_3-1, and cbb_3-2 independently supported the growth of *P. aeruginosa*. The growth rate of QXCb2

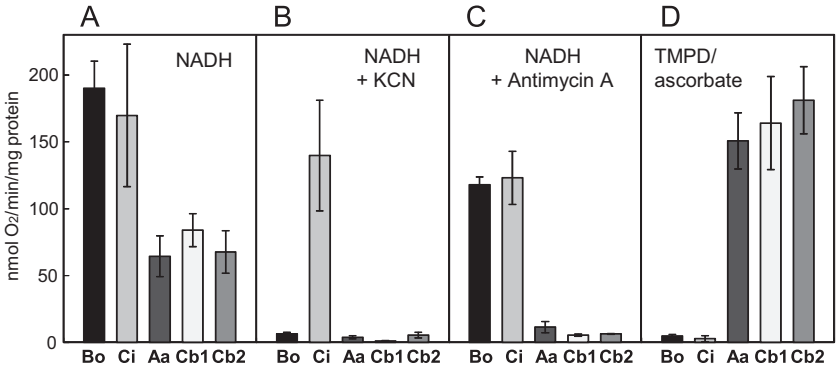

FIG 2 Oxygen consumption activities of the membrane fractions of the quadruple terminal oxidase mutant strains. Bo, Ci, Aa, Cb1, and Cb2 indicate membrane fractions of QXBo, QXCi, QXAaS2, QXCb1, and QXCb2, respectively. A 0.5 mM concentration of NADH (A, B, and C) and a 0.1 mM concentration of TMPD reduced by 5 mM ascorbate (D) were used as electron donors. A 1 mM concentration of KCN (B) or 10 μg/ml antimycin A (C) was added to the reaction mixture to inhibit the heme-copper oxidases or the cytochrome bc_1 complex, respectively. The data are means of results from three independent experiments. Error bars indicate standard deviations from the means.

under aerobic conditions was lower than that of QXCb1, QXBo, or QXCi (Fig. 1A). When Cyo or CIO was utilized as the terminal oxidase, the final OD_{600} was lower than that in the strains with only functional cbb_3-1 or cbb_3-2. Under microaerobic (2% O_2) conditions, QXCb1 and QXCb2 had growth rates similar to that of the wild-type strain, but QXBo and QXCi exhibited poor growth (Fig. 1B; see also Fig. S4B).

Although QXAa was unable to grow aerobically, a spontaneous suppressor mutant strain of QXAa, QXAaS2, which displayed slow growth under aerobic conditions in LB medium, was obtained after repeated aerobic subculturing. No mutation was found in the structural genes encoding aa_3 by nucleotide sequencing analysis of the *cox* genes of QXAaS2, indicating that aa_3 had the enzymatic ability to support the aerobic growth of *P. aeruginosa* (data not shown). QXAaS2 was therefore used for further enzymatic characterization of aa_3. The detailed isolation procedure and characterization of QXAaS2 will be published elsewhere.

Electron donors of terminal oxidases. aa_3, cbb_3-1, and cbb_3-2 are thought to function as cytochrome *c* oxidases, whereas Cyo and CIO have been characterized as quinol oxidases. Here, we examined the physiological electron donor for each terminal oxidase by measuring the oxygen consumption activity in the membrane fractions of each mutant strain grown under aerobic conditions (Fig. 2). When NADH was used as an electron donor, all strains that expressed only one terminal oxidase showed high oxygen consumption activity. The activities of QXBo and QXCi were higher than those of QXAaS2, QXCb1, and QXCb2 (Fig. 2A). Strain PTO5 grown under anaerobic denitrification conditions had no detectable oxygen consumption activity (data not shown). When 1 mM cyanide was added to the reaction mixture, only QXCi displayed high activity, indicating that CIO exclusively mediates cyanide-insensitive respiration under the tested conditions (Fig. 2B). Antimycin A, which is an inhibitor of the cytochrome bc_1 complex, significantly inhibited the oxygen consumption activities of QXAaS2, QXCb1, and QXCb2 but had only a minimal effect on QXBo and QXCi (Fig. 2C). In the presence of ascorbate-reduced TMPD, which specifically reduces cytochrome *c* and was used as an artificial electron donor, QXAaS2, QXCb1, and QXCb2 showed high oxygen-reducing activities, whereas QXBo and QXCi had almost no detectable activity (Fig. 2D). Together, these results clearly indicated that aa_3, cbb_3-1, and cbb_3-2 function as cytochrome *c* oxidases and that Cyo and CIO function as quinol oxidases *in vivo*.

The genome of *P. aeruginosa* contains several cytochrome *c* genes. In previous and unpublished microarray experiments, we showed that *cc4* (PA5490) and *cycB* (PA5300), encoding cytochromes c_4 and c_5, respectively, and PA4571 and PA5491, encoding uncharacterized cytochromes *c*, are expressed at relatively high levels (21). *cc4* and PA5491 were constitutively expressed probably as an operon. *cycB* was largely expressed in the exponential phase, and its expression level decreased in the stationary phase. PA4571 showed ANR-dependent expression. To identify which cytochromes *c* are utilized as physiological electron donors for cbb_3-1 and cbb_3-2, these four cytochrome *c* genes were disrupted in the mutant strains QXCb1 and QXCb2, and the growth profiles of the resulting mutants were investigated (Fig. 3A and D). Disruption of *cycB* or PA4571 had no effect on the growth rates of either of QXCb1 and QXCb2; however, disruption of *cc4* or PA5491 severely or slightly inhibited, respectively, the growth of these strains. The impaired growth of the *cc4* mutants of QXCb1 and QXCb2 was restored by the introduction of a plasmid expressing *cc4* (Fig. 3B and E). The poor growth of the PA5491 mutants was also restored by complementation with *cc4* but not with PA5491 (Fig. 3C and F). These results indicated that the two cbb_3-type oxidases preferentially utilize cytochrome c_4 as an electron donor *in vivo* under the tested conditions. As PA5491 is located upstream of *cc4* and is predicted to be transcribed with *cc4* as part of an operon, disruption of PA5491 might affect the expression of *cc4*. The impaired growth of the *cc4* mutants was not due to a deficiency in the function of other enzymes that use cytochrome c_4 as an electron donor or acceptor, because the growth of these mutants was restored by complementation with the *cioAB* genes encoding the alternative quinol oxidase CIO (Fig. 3B and E).

Oxygen affinity of terminal oxidases. The K_m values of the terminal oxidases for oxygen were determined by the amperometric method using an oxygen electrode and by the spectrophotometric method with myoglobin or leghemoglobin as oxygen reporters (Table 2). Plots of the representative results for both methods are shown in Fig. S1, S2, and S3 in the supplemental material. The K_m value of aa_3 could not be determined using myoglobin, because oxymyoglobin was immediately oxidized to metmyoglobin when the membrane fraction of QXAaS2 was added to

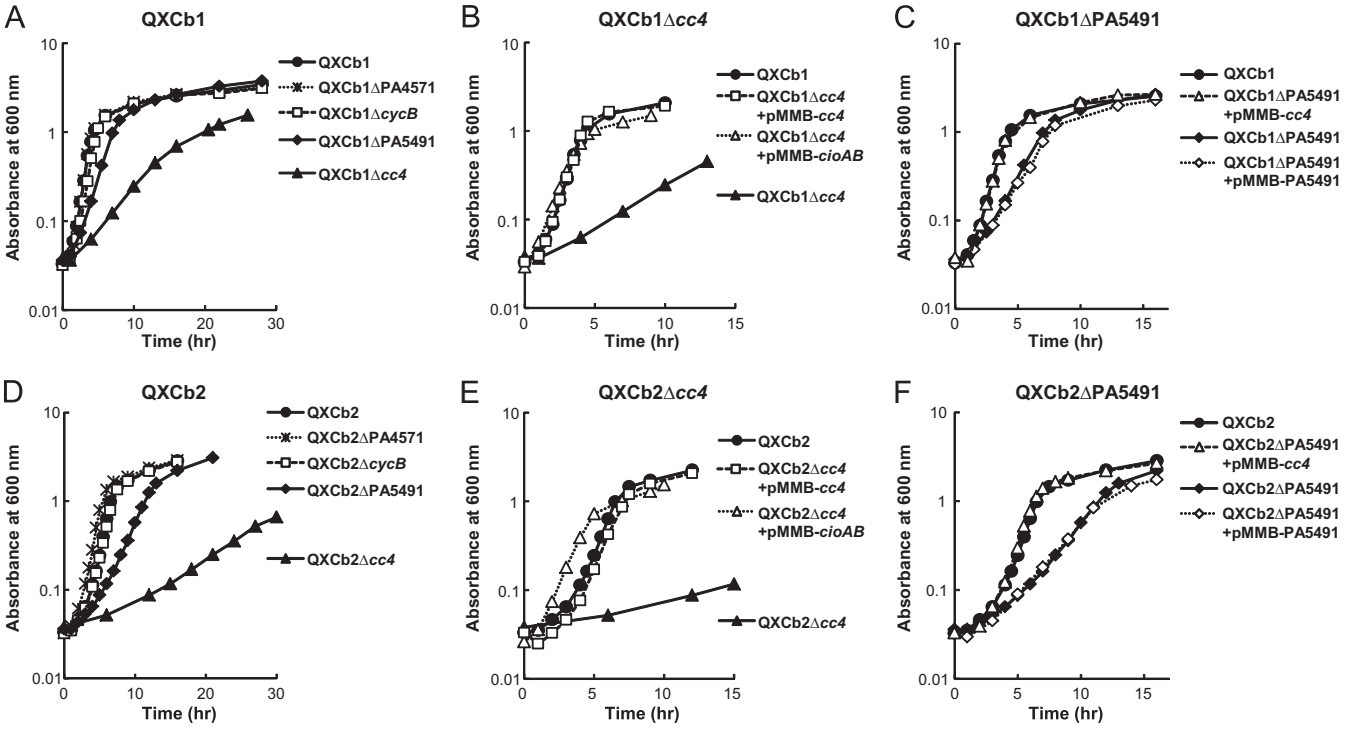

FIG 3 Effect of knockout of the cytochrome *c* genes on growth rates. Growth profiles of the derivatives of QXCb1 (A) and QXCb2 (D). Complementation of QXCb1Δ*cc4* (B), QXCb1ΔPA5491 (C), QXCb2Δ*cc4* (E), and QXCb2ΔPA5491 (F). The strains were grown aerobically in 40 ml LB medium in 100-ml Erlenmeyer flasks with shaking at 200 rpm. The data shown are representatives from three independent experiments. The same plots in linear scale are shown in Fig. S5 in the supplemental material.

the reaction mixture. The K_m values determined by measurements using an oxygen electrode were significantly higher than those determined using myoglobin, likely because of the relatively low sensitivity and slow response of the electrode to change in oxygen concentration (41). Although the oxygen electrode data underestimated the actual affinity of the terminal oxidases for oxygen, the relative difference of the estimated K_m values was similar to that of the spectrophotometric data. Relative comparisons of the data showed that the K_m values of Cyo, CIO, and aa_3 were similar to each other and higher than those of cbb_3-1 and cbb_3-2, clearly indicating that Cyo, CIO, and aa_3 are low-affinity enzymes.

The K_m values of cbb_3-1 and cbb_3-2 for oxygen did not significantly differ and were approximately 1 order of magnitude lower than those of the other three enzymes when evaluated by either method (Table 2). The K_m values of cbb_3-1 and cbb_3-2 determined by the myoglobin method were comparable to the reported values of the cbb_3-type oxidases from *B. japonicum* (7 nM) and *C. jejuni* (40 nM) (14, 28), indicating that both cbb_3-1 and cbb_3-2 are high-affinity enzymes. However, because these values were lower than the working range (0.1 to 10 μM) for myoglobin (37), we also determined the K_m values of cbb_3-1 and cbb_3-2 using partially purified pea nodule leghemoglobin (working range, 3 to 100 nM). The determined K_m values for cbb_3-1 and cbb_3-2 using leghemoglobin were 6.6 and 6.5 nM, respectively. However, the experiments using leghemoglobin were performed only once due to the limited amount of leghemoglobin that was available.

CIO of *P. aeruginosa* was previously predicted to have high affinity for oxygen (15). However, the K_m value of CIO for oxygen

TABLE 2 Affinity of terminal oxidases for oxygen[a]

	Oxygen electrode		Myoglobin		Leghemoglobin	
Terminal oxidase[b]	K_m (μM)	V_{max} (nmol/min/mg)	K_m (μM)	V_{max} (nmol/min/mg)	K_m (nM)	V_{max} (nmol/min/mg)
bo_3	3.2 ± 1.3	117 ± 14	0.25 ± 0.04	92 ± 13	ND[c]	ND
CIO	4.0 ± 2.1	213 ± 34	0.41 ± 0.10	130 ± 34	ND	ND
aa_3	4.3 ± 1.0	91 ± 38	ND	ND	ND	ND
cbb_3-1	0.25 ± 0.02	77 ± 30	0.044 ± 0.022	51 ± 15	6.6	7.7
cbb_3-2	0.23 ± 0.08	82 ± 35	0.032 ± 0.021	53 ± 25	6.5	8.5

[a] The K_m values for oxygen were determined using an oxygen electrode or deoxygenation kinetics of oxymyoglobin or oxyleghemoglobin. Values for the oxygen electrode and myoglobin methods are presented as means ± standard deviations of results from at least three independent experiments. Values for the leghemogbobin method were determined from only one experiment.

[b] Membrane fractions of the strains QXBo, QXCi, QXAaS2, QXCb1, and QXCb2 were used for determination of the oxygen affinity of bo_3, CIO, aa_3, cbb_3-1, and ccb_3-2 oxidases, respectively.

[c] ND, not determined.

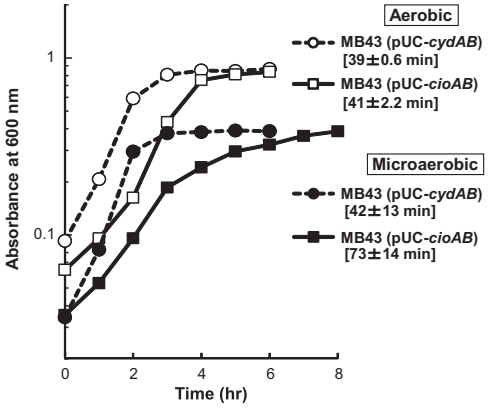

FIG 4 Functional heterologous expression of CIO in *E. coli* MB43, which lacks type 1 NADH dehydrogenase and three quinol oxidases. MB43 was transformed with pUC-*cioAB* or pUC-*cydAB*, and the transformants were cultivated in 100 ml LB medium in 300-ml square bottles. The medium was bubbled with air at a gas flux rate of 500 ml/min for aerobic conditions or with a gas mixture consisting of 2% O_2 and 98% N_2 at a gas flux rate of 150 ml/min for microaerobic conditions. The data shown are representatives from two independent experiments. The doubling times ± standard deviations are indicated in brackets. The same plots in linear scale are shown in Fig. S6 in the supplemental material.

measured here (Table 2) and the observed growth profile of QXCi under microaerobic conditions (Fig. 1B) clearly showed that *P. aeruginosa* CIO is a low-affinity enzyme, similar to the CIOs of *C. jejuni* and *G. oxydans* (13, 14). The low affinity of these CIOs is in contrast to the high affinity of the canonical *bd* oxidase of *E. coli* (42). To identify the functional difference between *P. aeruginosa* CIO and the canonical *bd* oxidase of *E. coli*, the *P. aeruginosa cioAB* genes or *E. coli cydAB* genes, which encode cytochrome *bd* oxidase 1, were transformed into *E. coli* MB43 (43), which lacks all three terminal oxidases and proton-pumping NADH dehydrogenase (NDH), and the growth profiles of the recombinant strains, MB43(pUC-*cioAB*) and MB43(pUC-*cydAB*), were compared under different oxygen conditions. MB43 does not grow by aerobic respiration because it has no proton-pumping activity, but aerobic growth was restored by the introduction of *cioAB*, indicating that CIO was functional in *E. coli* (Fig. 4; also see Fig. S6 in the supplemental material). Although the lag period of MB43(pUC-*cioAB*) under aerobic conditions was longer than that of MB43(pUC-*cydAB*), the maximum growth rates of the two strains were nearly identical. In contrast, the growth rate of MB43(pUC-*cioAB*) under microaerobic conditions was lower than that of MB43(pUC-*cydAB*). These results clearly showed that CIO was not optimally functional under low-oxygen conditions.

Proton translocation efficiency of the ETC of terminal oxidases. Aerobic respiration utilizes the proton motive force for ATP synthesis. Therefore, the ATP generation efficiency of aerobic respiration closely correlates with the efficiency of the electron transport chain (ETC) to create a proton gradient across the cell membrane. Protons are translocated across the cell membrane during the transfer of electrons through NDH, cytochrome bc_1 complex (complex III), and terminal oxidases. As *P. aeruginosa* has proton-pumping type I NDH (NDH-1) and non-proton-pumping type II NDH (NDH-2), the net number of protons translocated across the cell membrane per oxygen atom consumed (H^+/O ratio) depends on the proportion of NDH-1 and NDH-2 and the composition of terminal oxidases. Here, the H^+/O ratio was determined by the oxygen pulse method using a cell suspension of each mutant to evaluate the contribution of each terminal oxidase for generation of ATP. The H^+/O ratios of the ETCs terminated by Cyo, CIO, aa_3, cbb_3-1, and cbb_3-2 were approximately 4, 3, 6, 4, and 4, respectively (Table 3).

To compare the proton translocation stoichiometry of CIO with that of canonical cytochrome *bd* oxidase, the H^+/O ratios of *E. coli* MB43(pUC-*cioAB*) and MB43(pUC-*cydAB*) were also determined. Because MB43 does not have NDH-1, the values represent the H^+/O ratios of the quinol oxidase enzymes. The H^+/O ratio of CIO in *E. coli* MB43 was approximately 2, a ratio that was nearly identical with that of the *bd* oxidase 1 of *E. coli* (Table 3). This result indicated that the proton translocation stoichiometry of CIO was identical with that of the canonical cytochrome *bd* oxidase, irrespective of their differing affinities for oxygen.

DISCUSSION

P. aeruginosa has two tandemly located *cco* gene clusters encoding the cbb_3-type cytochrome *c* oxidases cbb_3-1 and cbb_3-2. The former is constitutively expressed, whereas the latter is induced under low-oxygen conditions and during the stationary growth phase (15, 17, 21). In the present study, we demonstrated that the growth rates of the quadruple terminal oxidase mutant strains QXCb1 and QXCb2, which expressed only cbb_3-1 or cbb_3-2, respectively, were comparable to or slightly higher than that of the wild-type strain under aerobic and microaerobic conditions, respectively (Fig. 1; see also Fig. S4 in the supplemental material). These results indicate that cbb_3-1 and cbb_3-2 are the main functioning terminal oxidases in *P. aeruginosa* under aerobic and microaerobic conditions, respectively, although the two enzymes have similar high affinities for oxygen (Table 2). High-affinity terminal oxidases are typically induced under low-oxygen conditions in many bacterial species and are often repressed under high-oxygen conditions, in which low-affinity terminal oxidases are the dominant enzymes (5, 44). Thus, the finding that high-affinity terminal oxidases predominantly function in *P. aeruginosa* even under high-oxygen conditions indicates that this is a characteristic feature of this ubiquitous organism.

Many pseudomonads, including *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Pseudomonas stutzeri*, have two sets of gene clusters encoding cbb_3 oxidases corresponding to cbb_3-1 and cbb_3-2, suggesting that carrying these two isoforms is advantageous for survival. Recently, Xie et al. (45) purified and characterized the two cbb_3 isoforms from *P. stutzeri* and found that they

TABLE 3 Proton translocation efficiency of whole cells expressing only one terminal oxidase

Strain	Terminal oxidase	H^+/O ratio[a]
QXBo	bo_3	4.04 ± 0.190
QXCi	CIO	2.83 ± 0.211
QXAaS2	aa_3	6.28 ± 0.281
QXCb1	cbb_3-1	4.07 ± 0.134
QXCb2	cbb_3-2	4.16 ± 0.235
MB43(pUC-*cioAB*)	CIO	1.85 ± 0.111
MB43(pUC-*cydAB*)	*E. coli bd* oxidase 1	2.04 ± 0.176

[a] The H^+/O ratio was determined by the oxygen pulse method. The aerobically grown stationary-phase cells were used. Values are means ± standard deviations of results from at least three independent experiments.

differed in thermal stability but did not have significant difference in the UV-visible spectrum or oxygen reductase and catalase activities. Here, we also found no significant difference in oxygen affinity or proton translocation efficiency between the two cbb_3 isoforms of *P. aeruginosa*. In addition, both cbb_3-1 and cbb_3-2 utilized cytochrome c_4 as the main electron donor under the tested aerobic conditions in rich medium. However, a difference was found between the aerobic growth profiles of strains QXCb1 and QXCb2, which express only cbb_3-1 and cbb_3-2, respectively, as the growth rate of QXCb2 was lower than that of QXCb1 (Fig. 1A; see also Fig. S4A in the supplemental material). The main reason for the difference was likely due to the low expression level of cbb_3-2 during the exponential phase (21), but it also is possible that cbb_3-2 is sensitive to high intracellular oxygen tension or oxidative stress.

The ETCs of *P. aeruginosa* terminated by cbb_3-1 and cbb_3-2 had nearly identical proton translocation stoichiometry (Table 3). Compared to type A family enzymes, cbb_3 oxidases have only one of two possible proton-conducting channels (46), and their proton-pumping efficiency remains a point of debate (40, 47). The H^+/O ratios determined in the present study were reflective of all active ETCs using endogenous electron donors. When respiratory chain inhibitors and artificial electron donors were added in our experimental system, clear results could not be obtained, likely due to the low permeability of the outer membrane of *P. aeruginosa* cells or the presence of endogenous electron donors. Therefore, the enzymatic proton-pumping efficiency of each terminal oxidase could not be determined. Although the H^+/O ratio of the ETC might be affected by the proportion of electron flow through NDH-1 and NDH-2 and the constitution of endogenous electron donors, the present results clearly indicate that *P. aeruginosa* cells produce less energy when cbb_3 oxidases are utilized than when aa_3 is utilized (Table 3).

aa_3 is a close phylogenetic relative of the mitochondrial terminal oxidase. The ETC of aa_3 generated a proton gradient across the cell membrane with the highest efficiency of all five examined terminal oxidases, indicating that aa_3 most efficiently generates ATP per molecule of nutrient (Table 3). This result is in good agreement with the fact that aa_3 is induced under nutrient starvation conditions (21). However, it was unexpected that the quadruple mutant strain QXAa was unable to grow aerobically (Fig. 1A), because aa_3-type oxidases play major roles in aerobic growth in many bacterial species (23–26). Transcription of the *cox* genes encoding aa_3 is repressed by the two-component regulator RoxSR and is dependent on the stationary-phase sigma factor RpoS (21). RoxSR is homologous to RegBA/PrrBA-type regulators, which regulate the transcription of photosynthesis genes of purple nonsulfur photosynthetic bacteria and are speculated to sense the electron flow of ETC or the redox status of the quinone pool (48, 49). One reason for the inability of strain QXAa to grow under aerobic conditions is that the *cox* genes may be tightly repressed in nutrient-rich LB medium.

The quinol oxidase Cyo was found to have low affinity for oxygen (Table 2). The gene arrangement and sequence of Cyo are similar to those of the *E. coli* bo_3 quinol oxidase, which is the main terminal oxidase in *E. coli* under aerobic conditions. The bo_3 oxidase also plays a major role under aerobic conditions in *P. putida*, which is a nonpathogenic relative of *P. aeruginosa* that also has five terminal oxidases corresponding to those of *P. aeruginosa* (50). In *P. putida*, knockout of the *cyo* genes significantly changes the transcriptome profile and relieves catabolite repression of the phenol and alkane degradation genes (51–53). In contrast to *P. putida*, Cyo of *P. aeruginosa* seems to play a minor role under normal growth conditions in LB medium because the expression level of the *cyo* genes is low (21) and aerobic growth is not affected in QXCb1, which expresses cbb_3-1 but lacks Cyo (Fig. 1). This difference in the major terminal oxidases of *P. aeruginosa* and *P. putida* might reflect the unique characteristics of each species. The *cyo* genes are upregulated in *P. aeruginosa* by the nitric oxide (NO)-generating reagent *S*-nitrosoglutathione (GSNO) or by iron starvation, indicating that Cyo is utilized under these stress conditions.

The copper-free quinol oxidase CIO belongs to the cytochrome *bd* family but is phylogenetically and characteristically distinct from the canonical *bd* oxidases (9–14). The amino acid sequences of the two subunits of CIO, CioA and CioB, are highly similar to those of the canonical *bd* oxidases CydA and CydB, respectively, but the conserved sequence of the periplasmic loop (Q loop) that contains the putative quinol-oxidizing site is significantly shorter in CioA than in CydA (9). CIO of *P. aeruginosa* was predicted to have high affinity for oxygen, but its K_m value for oxygen determined in this study was comparable to those of aa_3 and Cyo, indicating that it is a low-affinity enzyme, similar to the CIOs of *C. jejuni* and *G. oxydans* (Table 2) (13, 14). The low affinity of CIOs for oxygen is in contrast to the high affinity of the canonical *bd* oxidase of *E. coli* (42, 43). The growth rate of strain QXCi under the microaerobic conditions was lower than those of the strains that expressed the cbb_3 oxidases (Fig. 1B). It was also reported that CIO did not support the growth of *P. aeruginosa* under 0.4% O_2 conditions (15), indicating that CIO does not function under very low O_2 conditions. A *cco1 cco2* double mutant was able to grow under 2% O_2 conditions, but a *cco1 cco2 cio* triple mutant did not grow under the conditions in synthetic medium (15). The inability of the triple mutant to grow under the conditions was probably because of the sensitivity of Cyo and aa_3 to cyanide, which would be produced under low-O_2 conditions. However, Cyo was able to support the growth under 2% O_2 conditions in LB medium in the present study (Fig. 1B; see also Fig. S4B in the supplemental material). One of the reasons for the contradictory results might be the different stress resistances of the cells grown in different media.

Despite differences in oxygen affinities, the proton translocation stoichiometry of CIO was nearly identical with that of the *E. coli bd* oxidase (Table 3). The *bd* oxidases do not pump protons, but the oxidation of the quinol at the periplasmic side of the membrane and subsequent uptake of protons from the cytoplasm for formation of water result in the H^+/O stoichiometry of 2 (43). The H^+/O ratio of the entire ETC terminated by CIO was lowest among the ETCs involving the other terminal oxidases, indicating that the utilization of CIO is not efficient for the generation of ATP. CIO is induced by inhibitors of the respiratory chain, cyanide and sodium nitroprusside (SNP), or by copper starvation (16, 18, 19, 21). Thus, CIO acts as a complementary enzyme when the heme-copper oxidases are not functioning or are inhibited. The low proton translocation efficiency of CIO might also be advantageous to *P. aeruginosa* for maintaining the redox balance of the ETC when the respiratory electron flow is not consistent.

Conclusions and perspectives. Modulating the expression of multiple terminal oxidases with unique properties likely contributes to survival and proliferation of *P. aeruginosa* in diverse envi-

ronmental niches. In this study, we characterized the enzymatic properties of the five known terminal oxidases. The type A heme-copper superfamily enzymes, aa_3 and Cyo, had low affinity for oxygen. The aa_3 and bo_3 oxidases typically play a major role in respiration under aerobic conditions in many bacterial species (23–26, 50, 54). However, aa_3 and Cyo are specifically utilized in *P. aeruginosa* under starvation and specific stress conditions, respectively. Expression of aa_3 under starvation conditions is consistent with the fact that the ETC terminated by aa_3 has the highest energy generation efficiency among the five terminal oxidases examined here. The non-heme-copper oxidase CIO was also found to have low affinity for oxygen, and its energy generation efficiency was identical to that of the canonical *bd* oxidase. *P. aeruginosa* has two cbb_3 oxidases, cbb_3-1 and cbb_3-2, which belong to the type C family of heme-copper oxidases. Although both oxidases had high affinity for oxygen and the two were functionally similar, cbb_3-1 and cbb_3-2 played major roles in respiration under aerobic and microaerobic conditions, respectively. Utilization of cbb_3-1 as the main terminal oxidase under aerobic conditions may contribute to the resistance of *P. aeruginosa* to reactive oxygen species. Identification of the enzymatic differences between the two cbb_3 oxidase isoforms should be a future target for investigation.

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