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Expression of multiple cbb_3 cytochrome *c* oxidase isoforms by combinations of multiple isosubunits in *Pseudomonas aeruginosa*

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The ubiquitous opportunistic human pathogen *Pseudomonas aeruginosa* has five terminal oxidases for aerobic respiration and uses them under different growth conditions. Two of them are cbb_3-type cytochrome *c* oxidases encoded by the gene clusters *ccoN1O1Q1P1* and *ccoN2O2Q2P2*, which are the main terminal oxidases under high- and low-oxygen conditions, respectively. *P. aeruginosa* also has two orphan gene clusters, *ccoN3Q3* and *ccoN4Q4*, encoding the core catalytic CcoN isosubunits, but the roles of these genes have not been clarified. We found that 16 active cbb_3 isoforms could be produced by combinations of four CcoN, two CcoO, and two CcoP isosubunits. The CcoN3- or CcoN4-containing isoforms were produced in the WT cell membrane in response to nitrite and cyanide, respectively. The strains carrying these isoforms were more resistant to nitrite or cyanide under low-oxygen conditions. These results indicate that *P. aeruginosa* gains resistance to respiratory inhibitors using multiple cbb_3 isoforms with different features, which are produced through exchanges of multiple core catalytic isosubunits.

cbb_3 | terminal oxidase | cytochrome *c* oxidase | respiration | *Pseudomonas aeruginosa*

The cbb_3-type cytochrome *c* oxidase (cbb_3) is a bacteria-specific terminal oxidase of the heme-copper oxidoreductase superfamily that catalyzes the four-electron reduction of molecular oxygen to water at the end of the aerobic respiratory chain (1). cbb_3 has an extremely high affinity for oxygen and typically functions under low-oxygen conditions in many bacteria, including several pathogens of *Helicobacter*, *Campylobacter*, and *Neisseria* species (2–4). cbb_3 consists of four subunits that are encoded by the *ccoNOQP* operon. CcoN is the core catalytic subunit, and it contains a reaction center. CcoO and CcoP are transmembrane monoheme and diheme cytochromes *c*, respectively (5). CcoQ is known to affect the stability of the cbb_3 complex, but it is not necessarily a component of purified cbb_3 (6–8).

The ubiquitously distributed opportunistic human pathogen *Pseudomonas aeruginosa* has two cbb_3-type isoforms, cbb_3-1 and cbb_3-2, which are encoded by *ccoN1O1Q1P1* and *ccoN2O2Q2P2*, respectively. It also has three low-affinity enzymes: the bo_3-type quinol oxidase, cyanide-insensitive oxidase (CIO), and aa_3-type cytochrome *c* oxidase (9–11). These terminal oxidases are differentially regulated under various growth conditions, and they contribute to the bacterium's ability to reside in a wide variety of environmental niches. *P. aeruginosa* is unique in its utilization of the high-affinity cbb_3 oxidases as the main terminal oxidases both under high- and low-oxygen conditions. The *ccoN1O1Q1P1* cluster is constitutively expressed, whereas the *ccoN2O2Q2P2* cluster is up-regulated under low-oxygen conditions or at the stationary phase (12). *P. aeruginosa* also possesses two orphan *ccoNQ* gene clusters designated *ccoN3Q3* (PA1856-PA1855) and *ccoN4Q4* (PA4133-PA4134) (Fig. 1*A*). These gene clusters cannot produce active enzymes independently, and their functions remain unknown. In this present study, we investigated the function and expression profiles of the *ccoN3Q3* and *ccoN4Q4* gene clusters and found that the gene products could produce active cbb_3 isoforms via combinations with CcoO1 or CcoO2 and CcoP1 or CcoP2.

Results

Production of Active cbb_3 Complexes by the Orphan *ccoNQ* Gene Products. *P. aeruginosa* has four *cco* gene clusters, *ccoN1O1Q1P1*, *ccoN2O2Q2P2*, *ccoN3Q3*, and *ccoN4Q4*, which can produce four CcoN, two CcoO, two CcoP, and four CcoQ isosubunits. Because amino acid sequence identities between the isosubunits of CcoN, CcoO, and CcoP are relatively high (Table S1), we speculated that 16 isoforms of the cbb_3 enzyme could be produced via different combinations of the isosubunits (Fig. 1*A*). To examine the possibility, we constructed 16 expression plasmids that could produce combinations of the CcoN, CcoO, and CcoP isosubunits under the control of the *lac* promoter (Fig. S1*A*). The septuple mutant strain STO7, which lacks seven terminal oxidase gene clusters, was constructed and used as a host to express one of the cbb_3 isoforms as the only terminal oxidase. STO7 was unable to grow under aerobic conditions because of the lack of the active terminal oxidases. The aerobic growth of STO7 was restored via transformation with any of the expression plasmids (Fig. S1 *B* and *C*). The cytochrome *c* oxidase activity of the isoforms was also confirmed via the Nadi assay (Fig. 1*B*), which is a method used to detect cytochrome *c* oxidase-dependent respiration using *N*, *N*-dimethyl-*p*-phenylenediamine as an artificial electron donor (13). These results clearly indicate that the functional cbb_3 isoforms could be produced in *P. aeruginosa* via any combination of the isosubunits. The isoforms could be classified to

Significance

The cbb_3 cytochrome *c* oxidase has high affinity for oxygen and is typically used for respiration in many pathogenic bacteria in hypoxic environments. Here we show that the opportunistic human pathogen *Pseudomonas aeruginosa* has a capability to produce 16 different cbb_3 isoforms by combinations of multiple isosubunits. Utilization of multiple isoforms with different properties contributes to the robustness of the bacterium under hypoxic growth conditions and might have practical implications for resistance to antibiotics and formations of biofilms and persister cells. Our findings indicate that the mechanism for the production of the isoforms could be possible therapeutic targets for treating chronic *P. aeruginosa* infection.

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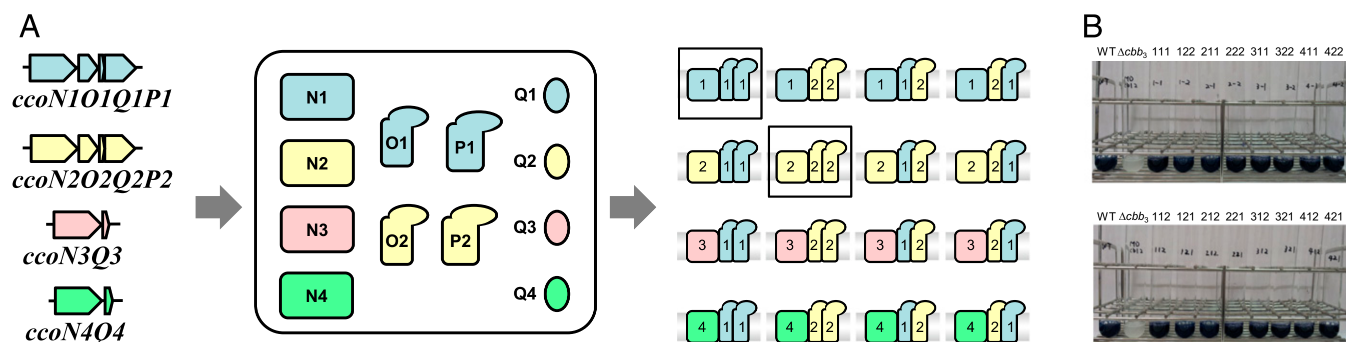

Fig. 1. Multiple cbb_3 isoforms in *P. aeruginosa*. (*A*) Sixteen isoforms of cbb_3 could be produced via combinations of the isosubunits encoded by four *cco* gene clusters. cbb_3-1 and cbb_3-2 are shown in boxes. (*B*) Cytochrome *c* oxidase activity of the isoforms visualized in blue color by the Nadi assay. WT and Δcbb_3 indicate PAO1 and the *ccoN1O1Q1P1 ccoN2O2Q2P2* double-mutant MCb12, respectively. Triple-digit numbers indicate the combination of the CcoN, CcoO, and CcoP isosubunits. For example, 111, 212, 321, and 422 indicate CcoN1O1P1, CcoN2O1P2, CcoN3O2P1, and CcoN4O2P2, respectively. The isoforms were expressed in strain STO7 with the expression plasmids shown in Fig. S1.

N1, N2, N3, and N4 types based on the isotypes of the core catalytic CcoN subunit.

Expression Profiles of the *ccoN3* and *ccoN4* Promoters. We explored the conditions under which *ccoN3Q3* and *ccoN4Q4* were up-regulated (Fig. 2). Previous microarray experiments illustrated that *ccoN3Q3* was up-regulated under anaerobic denitrification conditions (14). Because nitrite, NO, and N_2O are produced as intermediates of denitrification, *ccoN3Q3* might be regulated by one of these nitrogen oxides or derived reactive nitrogen species (RNS). Activation of the *ccoN3* promoter under denitrification was confirmed with a promoter assay using the *lacZ* transcriptional fusion fragment (Fig. 2*A*). The promoter was not up-regulated by nitrate or the NO-generating reagent sodium nitroprusside, but nitrite significantly activated the *ccoN3* promoter under aerobic conditions. These results indicated that the induction signal for the *ccoN3* promoter was nitrite.

ccoN4Q4 is located in a gene cluster (PA4129-4134) reported to be inducible by cyanide (15). Activation of the *ccoN4* promoter by cyanide was confirmed by the *lacZ* assay (Fig. 2*B*). *ccoN4* exhibited high transcriptional activity at the stationary phase because *P. aeruginosa* produces cyanide during this phase (16). In the *hcnB* KO mutant, which does not produce cyanide, the *ccoN4* promoter activity was extremely low, but the activity was significantly induced by the addition of cyanide. These results demonstrated that the induction signal for the *ccoN4* promoter was cyanide.

Identification of the Types of cbb_3 Isoforms Expressed in the Cell Membrane. Expression of the N3- or N4-type isoforms in the cell membrane of the WT strain PAO1 was investigated by an immunological method using specific antibodies that recognize all CcoN isosubunits (anti–*N*-all), only CcoN3 (anti-N3), and only CcoN4 (anti-N4). The specificities of the antibodies were confirmed using the membrane fractions of the cells that expressed only one of the isoforms (Fig. S2 *A–C*). The CcoN isosubunits were detected at 35–37 kDa after SDS/PAGE separation, and no cross-reactivity of the anti-N3 and anti-N4 antibodies was observed. The calculated molecular weights of CcoN1, CcoN2, CcoN3, and CcoN4 were 53.1, 53.1, 53.7, and 52.3 kDa, respectively. The sizes of the detected bands on the SDS/PAGE gel were lower than the calculated values, probably because of the high hydrophobicity of CcoN isosubunits.

Two-dimensional (2D) blue native (BN)/SDS/PAGE was used to detect the CcoN isoforms that constituted the cbb_3 enzyme complex in the cell membranes. BN/PAGE is a method for separating multiprotein complexes under native conditions (17). After separation of the membrane fraction of the aerobically grown WT cells by first-dimension BN/PAGE, the fraction was further separated by second-dimension SDS/PAGE, and *c*-type cytochromes were visualized by heme staining (18) (Fig. S2*D*). Cytochromes *c* of ~32 and 25 kDa were detected by SDS/PAGE at ~220 and 270 kDa and lower than 60 kDa by BN/PAGE. These bands were not detected in the membrane fraction of strain MDCb12, which is a KO mutant of the *ccoN1O1Q1P1* and

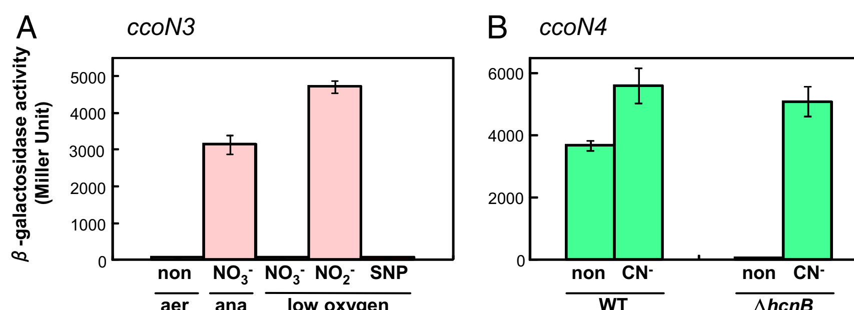

Fig. 2. Induction of the *ccoN3* and *ccoN4* promoters by nitrite and cyanide. (*A*) Promoter activity of *ccoN3*. The strain was cultured under aerobic (aer), anaerobic (ana), or 2% O_2 (low-oxygen) conditions. The medium was not supplemented (non) or supplemented with 40 mM $NaNO_3$ (NO_3^-), 20 mM KNO_2 (NO_2^-), or 3 mM sodium nitroprusside (SNP). The activities were measured at exponential phase. (*B*) Promoter activity of *ccoN4*. The strain was cultured under aerobic conditions. The medium was not supplemented (non) or supplemented with 300 μM KCN (CN^-). The activities were measured at early stationary phase. The promoter activity was measured as β-galactosidase activity using PAO1 (WT) or the *hcnB* mutant (Δ*hcnB*) carrying a transcriptional fusion of *ccoN3* (*A*) or *ccoN4* (*B*) with *lacZ*. Bars indicate means (error bars, SDs of at least two replicates).

ccoN2O2Q2P2 genes (Fig. S2*E*), indicating that the 32- and 25-kDa proteins corresponded to CcoP and CcoO, respectively. When the 2D gel of the WT cell membrane was used to detect the CcoN isosubunits by Western blotting with the anti-*N*-all antibody, the 35-kDa proteins corresponding to the CcoN isosubunits were detected at 220 and 270 kDa by BN/PAGE (Fig. 3*A*). These results indicated that the 220- and 270-kDa protein complexes separated by BN/PAGE contained the CcoN, CcoO, and CcoP subunits.

The active CcoNOQP complex of *Rhodobacter capsulatus* was reported to be separated at 230 kDa by BN/PAGE (19). The apparent molecular weight of the CcoNOP complexes of *Pseudomonas stutzeri* as determined by gel filtration chromatography was ∼190 kDa (20). Thus, the protein complex separated at 220 kDa by BN/PAGE was expected to be the CcoNOP or CcoNOQP complex. The complex was probably associated with additional proteins in the 270-kDa complex. The 32- and 25-kDa cytochromes *c* detected at lower than 60 kDa by BN/PAGE were probably the CcoP and CcoO proteins dissociated from the cbb_3 complexes (Fig. S2*D*). Free CcoN proteins were not detected, suggesting that CcoN was unstable without association with the CcoO and CcoP subunits. When the cells that were cultivated under the anaerobic denitrification conditions were used for the analysis, the cbb_3 complex was detected at ∼240 kDa, suggesting that the CcoNOP complex was associated with other protein in the anaerobic cells (Fig. S2 *F* and *G*). From these results, the CcoN isosubunits that constituted the cbb_3 isoforms via association with CcoO and CcoP were expected to appear at 220 and 270 kDa for the aerobic cells and at 240 kDa for the anaerobic cells by BN/PAGE and at 35–37 kDa by SDS/PAGE on 2D gels.

When lysates of exponential-phase WT cells cultured under aerobic conditions were tested on 2D gels, the anti-*N*-all antibody detected CcoN at the position of the cbb_3 complex, but the anti-N3 and anti-N4 antibodies detected no protein, indicating that only N1- and/or N2-type isoforms were expressed under the condition (Fig. 3 *A*–*C*). CcoN3 was detected when WT cells were cultured aerobically with nitrite or anaerobically via denitrification, but not in the *ccoN3*-mutant cells (Fig. 3 *D*–*F*). CcoN4 was detected in the exponential-phase WT cells cultured aerobically in the presence of cyanide or in the stationary-phase WT cells, but not in the *ccoN4*-mutant cells (Fig. 3 *G*–*I*). These results clearly indicated that CcoN3 or CcoN4 was associated with CcoO and CcoP in the WT cells under the conditions when the *ccoN3* or *ccoN4* promoter was induced, respectively.

Resistance of the CcoN3- and CcoN4-Containing Isoforms to Respiratory Inhibitors. Because nitrite and cyanide are known to inhibit cellular respiration, we examined the resistance of the isoforms to these compounds. The *ccoN3* mutant displayed no difference in growth from the WT irrespective of the presence of 20 mM nitrite under aerobic [20% (vol/vol) O_2] and anaerobic conditions. However, when the ambient oxygen concentration was 0.5%, the growth of the *ccoN3* mutant was slightly retarded compared with that of the WT (Fig. 4*A*). The strains that had only the N1- or N2-type isoform exhibited a longer lag period than the strains that had only the N3-type isoforms when 20 mM nitrite was added to the medium (Fig. 4*B*). These results indicate that the N3-type isoforms are more resistant to nitrite or derived RNS and function under low-oxygen conditions in the presence of nitrite.

The *ccoN4* mutation had no effect on the sensitivity to cyanide under aerobic conditions because of cyanide-resistant respiration mediated by CIO. However, when the ambient oxygen concentration was 0.5%, the *ccoN4* mutant exhibited sensitivity to cyanide (Fig. 4*C*). CIO may not be fully functional under extremely low-oxygen conditions because the affinity of CIO for oxygen is low (10). We examined the inhibitory effect of cyanide on the oxygen consumption activities of the isoforms (Fig. 4*D*). The 50% inhibition concentrations of CcoN1O1P1, CcoN2O2P2, CcoN4O1P1, and CcoN4O2P2 isoforms for cyanide were 0.23,

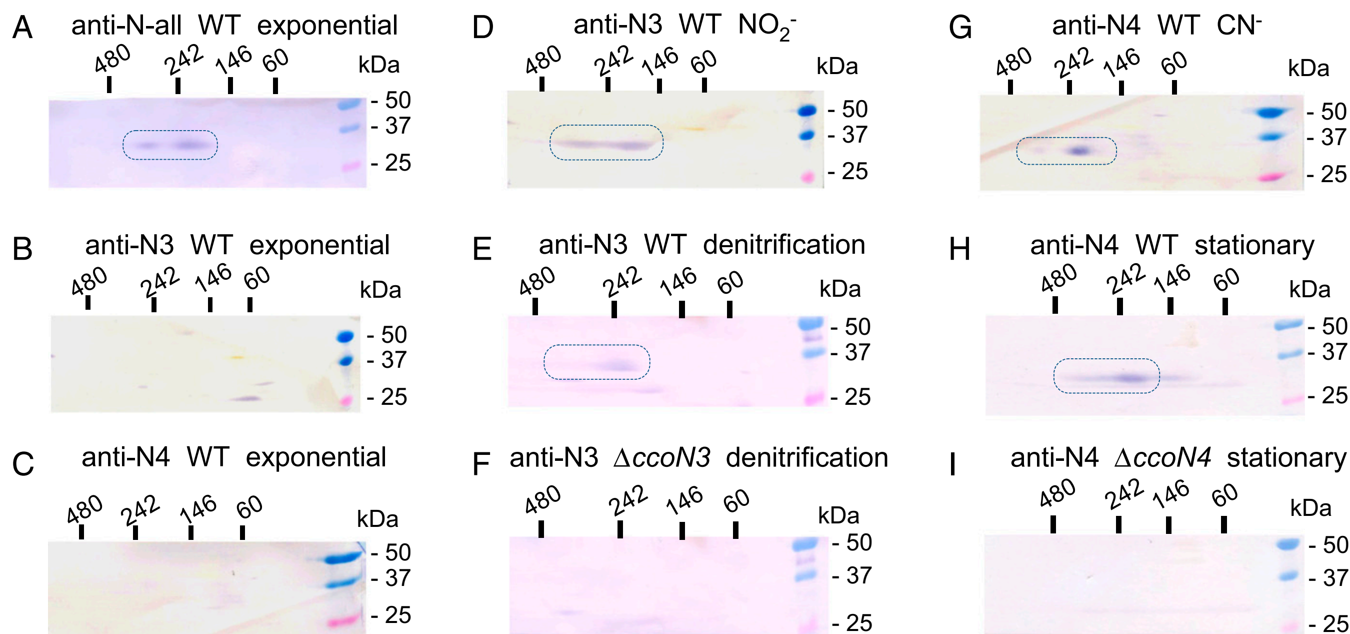

Fig. 3. Identification of the hybrid cbb_3 isoforms. The CcoN isosubunits that constituted the cbb_3 complexes were detected by Western blotting after solubilized membrane fractions (5–50 μg) were separated on 2D gels (horizontal, BN/PAGE; vertical, SDS/PAGE). Specific antibodies that detect all four CcoN isosubunits (*A*), only CcoN3 (*B and D*–*F*), and only CcoN4 (*C and G*–*I*) were used. The membrane fractions were prepared from the exponential-phase cells of PAO1 cultured aerobically in nonsupplemented LB medium (*A*–*C*) or medium supplemented with 20 mM KNO_2 (*D*) or 300 μM KCN (*G*); cells of PAO1 (*E*) and the *ccoN3* mutant (*F*) cultured anaerobically by denitrification; and the stationary-phase cells of PAO1 (*H*) and the *ccoN4* mutant (*I*) cultured aerobically in LB medium. The bands corresponding to the CcoN isosubunits in the cbb_3 complex are indicated by rounded rectangles.

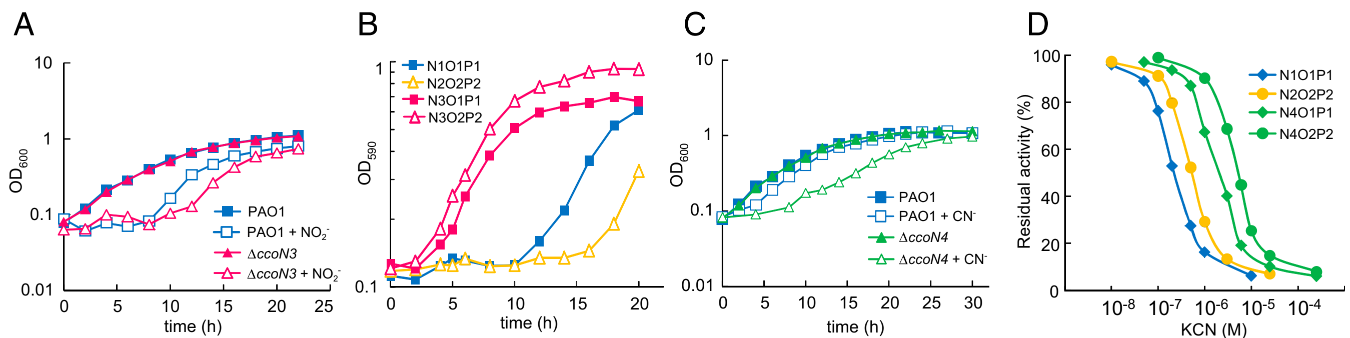

Fig. 4. Resistance of the CcoN3- and CcoN4-containing cbb_3 isoforms to nitrite and cyanide. (*A*) Growth profiles of PAO1 and the *ccoN3* mutant in LB medium in the presence or absence of 20 mM KNO_2 under low-oxygen (0.5%) conditions. (*B*) Growth profiles of STO7 transformed with plasmids expressing CcoN1O1P1, CcoN2O2P2, CcoN3O1P1, or CcoN3O2P2. The strains were cultured aerobically in 96-well plates. (*C*) Growth profiles of PAO1 and the *ccoN4* mutant in LB medium in the presence or absence of 300 μM KCN under low-oxygen (0.5%) conditions. (*D*) Oxygen consumption activities of the membrane fraction of the cells of STO7 transformed with plasmids expressing CcoN1O1P1, CcoN2O2P2, CcoN4O1P1, or CcoN4O2P2. Ascorbate-reduced TMPD was used as an electron donor for the reaction. KCN was added at various concentrations in the reaction mixture. Values are shown as percentages of the maximal activity level in the absence of KCN. Data are representatives of at least two independent experiments.

0.52, 2.0, and 5.0 μM, respectively. The values of the N4-type isoforms were one order of magnitude higher than those of the N1- or N2-type isoforms, indicating that the N4-type isoforms are involved in cyanide-resistant respiration in low-oxygen environments.

Oxygen Affinity of the cbb_3 Isoforms. We previously reported K_m values of cbb_3-1 and cbb_3-2 using the cell membrane of quadruple mutants of *P. aeruginosa* (10). These mutants carried *ccoN3Q3* and *ccoN4Q4*. Because CcoN4 was expressed at the stationary phase, the cell membranes might contain the N4-type isoforms. We determined here K_m values of the isoforms for oxygen using the strains that had only one of the recombinant isoforms by a spectrophotometric method with myoglobin as an oxygen reporter (10) (Table 1). Because the recombinant isoforms were expressed from the *lac* promoter on the vector, the concentrations of the isoforms in the cell membranes were expected to be similar. All of the tested isoforms were found to have higher affinity for oxygen than the low-affinity enzymes aa_3, bo_3, and CIO (10). The N3-type isoforms had slightly lower affinity than those of the other types. The N3-type isoforms might acquire higher resistance to nitrite or RNS at the expense of affinity for oxygen.

Discussion

Many pseudomonads have two sets of *cco* gene clusters encoding cbb_3 oxidases of the N1 and N2 types. The isoforms corresponding to cbb_3-1 and cbb_3-2 of *P. aeruginosa* are oppositely designated as cbb_3-2 and cbb_3-1, respectively, in other *Pseudomonas* species, such as *Pseudomonas putida* and *P. stutzeri* (21, 22). Xie et al. reported that the two isoforms from *P. stutzeri* differed in thermal stability but had no significant difference regarding the UV-visible spectrum or enzymatic activities (22). The affinities of the N1- and N2-type isoforms for oxygen were not significantly different in *P. aeruginosa* or the difference was smaller than the experimental errors (Table 1). Because cbb_3-1 and cbb_3-2 are used as the main terminal oxidases under high- and low-oxygen conditions, respectively (12), they might have difference in the resistance and sensitivity to oxygen or ROS. The growth of the strain that had only cbb_3-2 (CcoN2O2P2) exhibited a significantly prolonged lag period when it was cultured aerobically (Fig. S1*B*). A long lag period was also observed for the quadruple mutant strain QXCb2, which lacks the genes encoding aa_3, bo_3, CIO, and cbb_3-1 oxidases (10). Growth retardation was not observed when the strain was cultured under low-oxygen conditions. cbb_3-2, but not the other N2-type isoforms, is probably highly sensitive to oxygen or ROS. cbb_3-1 must be more resistant to oxygen, and utilization of cbb_3-1 under the aerobic conditions might contribute to the robustness of *P. aeruginosa* by suppressing the generation of ROS, because the high-affinity cbb_3 oxidases scavenge submicromolar concentrations of oxygen.

Analysis of the reported genome sequences revealed that several *Pseudomonas* species have one or two orphan *ccoNQ* gene clusters corresponding to *ccoN3Q3* or *ccoN4Q4* (Fig. S3). The species that carry *ccoN3*-type genes use denitrification genes for anaerobic respiration. Nitrite is one of the intermediates of the denitrification pathway that is temporarily accumulated during growth under denitrification (23). The N3-type enzymes, which are resistant to nitrite or derived RNS, might be operative under hypoxic conditions when aerobic respiration and denitrification occur simultaneously. The resistance to RNS might also contribute to the virulence of *P. aeruginosa* because macrophages of the host immune system produce NO to counteract bacterial infections.

P. aeruginosa produces cyanide under low-oxygen conditions (16). Cyanogenesis is believed to contribute to the virulence of *P. aeruginosa* by suppressing the growth of other microorganisms infected in the same niches and by killing the host cells (24). The *Pseudomonas* species that carry the *ccoN4*-type genes were found to carry *hcn* genes involved in the biosynthesis of cyanide (25) (Fig. S3), suggesting that the expression of the N4-type isoforms, which have higher tolerance to cyanide, is advantageous for counteracting the endogenous cyanide in the cyanogenic pseudomonads.

The respiratory system of *P. aeruginosa* has been uncovered to be far more complex than was previously thought. It has a

Table 1. Affinity of the cbb_3 isoforms for oxygen

Isoforms	K_m (μM)	V_{max} (nmol O_2/min/mg-protein)
CcoN1O1P1	0.012 ± 0.003	11 ± 3
CcoN1O2P2	0.027 ± 0.003	24 ± 5
CcoN2O1P1	0.016 ± 0.006	16 ± 3
CcoN2O2P2	0.022 ± 0.005	34 ± 3
CcoN3O1P1	0.121 ± 0.024	25 ± 7
CcoN3O2P2	0.151 ± 0.039	21 ± 5
CcoN4O1P1	0.046 ± 0.015	15 ± 2
CcoN4O2P2	0.035 ± 0.015	52 ± 13

K_m values for oxygen were determined using the deoxygenation kinetics of oxymyoglobin. Values are presented as means ± SDs from three independent experiments. Membrane fractions of the cells of strain STO7 transformed with the plasmids expressing only one of the cbb_3 isoforms were used for determination of the oxygen affinity (Fig. S1).

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capability to produce 16 different cbb_3 isoforms. Although it is not clear which isoforms are present under different growth conditions, at least each ones of the N3- and N4-type isoforms were identified to be expressed in response to the respiratory inhibitors. *P. aeruginosa* causes life-threatening infection in the airways of patients with cystic fibrosis (CF). Because the mucus layer in the lungs of patients with CF is depleted of oxygen, the ability to survive and proliferate in hypoxic environments is important for chronic infection (14, 26). As mentioned previously, resistance to RNS and cyanide is advantageous for survival in hypoxic environments. Aerobic respiration under hypoxic environments is mediated by the high-affinity cbb_3 oxidases. We revealed that *P. aeruginosa* acquires resistance to these respiratory inhibitors by producing multiple cbb_3 isoforms with different features. The subunit switch mechanism for the production of the multiple cbb_3 isoforms would be a promising therapeutic target for treating chronic *P. aeruginosa* infection.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions. All bacterial strains, plasmids, and growth conditions used in this study are described in Table S2 and *SI Materials and Methods*. The primers used for PCR amplification of the DNA fragments, which were used for construction of the plasmids, are shown in Table S3.

Determination of Enzymatic Activities and Biochemical Parameters. Cytochrome *c* oxidase activity was visualized using the Nadi assay (13). Cells of overnight cultures were suspended in 1 mL 20 mM Tris·HCl (pH 7.5). After adding 200 μL of a 1:1 mixture of 35 mM α-naphthol in ethanol and 30 mM *N*,*N*-dimethyl-*p*-phenylenediamine monohydrochloride to the cell suspension, the development of blue color by the formation of indophenol blue was observed. The reaction mixtures were incubated for 5 min at room temperature. Oxygen consumption activity was determined amperometrically using an Apollo 4000 free radical analyzer equipped with a 2-mm ISO-OXY-2 O_2 electrode (WPI) according to the procedures described previously (10). The method for determination of the K_m values for oxygen is described in *SI Materials and Methods*.

Western Blotting and Heme Staining. Preparation of solubilized membrane fractions and gel electrophoresis are described in *SI Materials and Methods*. After separation of the solubilized membrane fraction by SDS/PAGE or 2D BN/SDS/PAGE, proteins were transferred to PVDF membranes (Sequi-Blot PVDF membrane; Bio-Rad) using a semidry blotting system (HorizBlot AE-6677P; ATTO). Polyclonal anti–*N*-all, anti-N3, and anti-N4 antibodies were raised against synthetic oligopeptides in rabbits. The sequences of the anti–*N*-all, anti-N3, and anti-N4 oligopeptides were C + MWRAVNEDGTLTYS, C + TLRSAQDQRQPVLA, and C + RTVRQARPEGIL, respectively. The oligopeptides and antisera were prepared by Eurofins Genomics. Goat anti-rabbit IgG, pAb HRP conjugate (Enzo Life Science) was used as the secondary antibody. The desired proteins were detected using a POD Immunostain Set (Wako). Cytochromes *c* in the 2D gels were visualized by heme-linked peroxidase staining with 3,3′,5,5′-tetramethylbenzidine as previously described (18).

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