

*cbb*₃-Type Cytochrome *c* Oxidases, Aerobic Respiratory Enzymes, Impact the Anaerobic Life of *Pseudomonas aeruginosa* PAO1

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For bacteria, many studies have focused on the role of respiratory enzymes in energy conservation; however, their effect on cell behavior is poorly understood. *Pseudomonas aeruginosa* can perform both aerobic respiration and denitrification. Previous studies demonstrated that *cbb*₃-type cytochrome *c* oxidases that support aerobic respiration are more highly expressed in *P. aeruginosa* under anoxic conditions than are other aerobic respiratory enzymes. However, little is known about their role under such conditions. In this study, it was shown that *cbb*₃ oxidases of *P. aeruginosa* PAO1 alter anaerobic growth, the denitrification process, and cell morphology under anoxic conditions. Furthermore, biofilm formation was promoted by the *cbb*₃ oxidases under anoxic conditions. *cbb*₃ oxidases led to the accumulation of nitric oxide (NO), which is produced during denitrification. Cell elongation induced by NO accumulation was reported to be required for robust biofilm formation of *P. aeruginosa* PAO1 under anoxic conditions. Our data show that *cbb*₃ oxidases promote cell elongation by inducing NO accumulation during the denitrification process, which further leads to robust biofilms. Our findings show that *cbb*₃ oxidases, which have been well studied as aerobic respiratory enzymes, are also involved in denitrification and influence the lifestyle of *P. aeruginosa* PAO1 under anoxic conditions.

Respiration is a fundamental process for energy conservation. In eukaryotic mitochondria, *aa*₃-type cytochrome *c* oxidase (terminal oxidase) catalyzes the terminal reaction of electron transport in aerobic respiration through oxygen reduction. Furthermore, the generation of reactive oxygen species (ROS) and the oxidation of cytochrome *c* by *aa*₃ oxidase can induce apoptosis; thus, *aa*₃ oxidase also plays a role in cell fate determination (1, 2). In bacteria, multiple terminal oxidases have been reported to catalyze energy conservation; however, few studies have reported their influence on cell behavior.

Pseudomonas aeruginosa is a ubiquitous bacterium with versatile aerobic and anaerobic respiratory systems (3). Under oxic environments, five terminal oxidases (Cyo, CIO, *aa*₃, *cbb*₃-1, and *cbb*₃-2) reduce oxygen as terminal electron acceptors. The terminal oxidases Cyo and CIO are quinol oxidases, while the terminal oxidases *aa*₃ and *cbb*₃ are cytochrome *c* oxidases. Compared with other terminal oxidases, *cbb*₃ oxidases possess a high affinity for oxygen. *cbb*₃-1 and *cbb*₃-2 oxidases are encoded by the tetracistronic *ccoNOQP-1* and *ccoNOQP-2* operons (4). The *ccoNOQP-1* operon is constitutively transcribed under various oxygen concentrations (5). On the other hand, the *ccoNOQP-2* operon is highly transcribed under low oxygen concentrations because the oxygen-responsive transcriptional regulator ANR upregulates its promoter activity. These enzymatic and transcriptional characteristics of *cbb*₃ oxidases enable *P. aeruginosa* to grow in low-oxygen environments. In addition, under anoxic conditions, where oxygen is depleted, *P. aeruginosa* can use N-oxides as alternative electron acceptors in a process known as denitrification. During this process, nitrate (NO₃⁻) is reduced subsequently to nitrite (NO₂⁻), nitric oxide (NO), nitrous oxide (N₂O), and, finally, dinitrogen (N₂). Each reduction step is performed by NO₃⁻ reductase (NAR), NO₂⁻ reductase (NIR), NO reductase (NOR), and N₂O reductase (NOS). The denitrification process is triggered by physicochemical factors, such as oxygen and N-oxides, via ANR and other transcriptional regulators, such as NarXL and DNR (6, 7). Moreover, quorum sensing (QS) molecules, such as N-acyl-L-homoserine lactone (AHL) and *Pseudomonas* quinolone

signal (PQS), fine-tune the metabolic activity to control the NO concentration (8, 9).

Interestingly, Kawakami et al. reported that *cbb*₃ oxidases of *P. aeruginosa* are more highly expressed under anoxic denitrifying conditions than are other terminal oxidases, suggesting that *cbb*₃ oxidases may have a unique role under anoxic conditions (5). However, their role has not yet been elucidated. In this study, we showed that *cbb*₃ oxidases of *P. aeruginosa* PAO1 lead to NO accumulation during the denitrification process under anoxic conditions. This effect resulted in the promotion of biofilm formation via cell elongation. Such a morphological change and complex formation are helpful to survival under many environmental stresses (10, 11). Thus, it is expected that *cbb*₃ oxidases are key enzymes involved in regulating these cell behaviors in response to anoxic environments. Our findings show a new role of *cbb*₃ oxidases, emphasizing their importance in bacterial physiology.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. For routine cultures, bacterial strains were grown at 37°C in lysogeny broth (LB) medium (Lennox; Nacalai, Kyoto, Japan) or on LB agar plates. When necessary, gentamicin was added at the concentration of 10 µg ml⁻¹ for *Escherichia coli* and 100 µg ml⁻¹ for *P. aeruginosa*. Anoxic planktonic cultures were prepared using a method

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>E. coli</i>		
JM109	<i>E. coli</i> strain for transformation	TaKaRa
DH5 α	<i>E. coli</i> strain for transformation	TaKaRa
S17-1	Mobilizer strain	14
<i>P. aeruginosa</i>		
PAO1	Wild type	36
Δ <i>ccoN1</i> mutant	PAO1 with a markerless deletion of <i>ccoN1</i> gene	This study
Δ <i>ccoN2</i> mutant	PAO1 with a markerless deletion of <i>ccoN2</i> gene	This study
Δ <i>ccoN1</i> Δ <i>ccoN2</i> mutant	PAO1 with markerless deletions of <i>ccoN1</i> and <i>ccoN2</i> genes	This study
Δ <i>nir</i> mutant	PAO1 with markerless deletions of <i>nirS</i> to <i>nirN</i> genes	15
Δ <i>ccoN1</i> Δ <i>ccoN2</i> Δ <i>nir</i> mutant	PAO1 with markerless deletions of <i>ccoN1</i> , <i>ccoN2</i> , and <i>nirS</i> to <i>nirN</i> genes	This study
PS1700	<i>narG</i> ; 3,835 bp deleted from PA3874 to PA3876	12
Plasmids		
pHSG398	Cloning vector; Cp ^r	TaKaRa
pG19II	pK19mobsac derived suicide vector; <i>sacB</i> Gm ^r	13
pG19ccoN1	<i>ccoN1</i> deletion cassette in pG19II	This study
pG19ccoN2	<i>ccoN2</i> deletion cassette in pG19II	This study
pG19nir	<i>nirS</i> -to- <i>nirN</i> deletion cassette in pG19II	15
pMEX9	pME4510 derived promoter-probe vector; <i>xylE</i> Gm ^r	8
pMEXccoN1	<i>ccoN1</i> promoter region in pMEX9	This study
pMEXccoN2	<i>ccoN2</i> promoter region in pMEX9	This study

^a Cp^r, chloramphenicol resistant; Gm^r, gentamicin resistant.

described previously (9). *P. aeruginosa* was grown oxically in 24-ml test tubes containing 4 ml of LB medium for 10 h and inoculated into butyl-rubber-sealed 17-ml Hungate tubes containing 5 ml of LB medium supplemented with 100 mM KNO₃ (LBN medium) at a starting optical density at 660 nm (OD₆₆₀) of 0.01. The air of the Hungate tubes was replaced with argon by flushing gas through a needle. Anoxic planktonic incubation was performed at 37°C with shaking at 200 rpm. By using a negative-control denitrification mutant (PS1700) (12), it was confirmed that this experimental condition is anoxic (see Fig. S1 in the supplemental material). Hypoxic incubation was carried out by using butyl-rubber-sealed 17-ml Hungate tubes containing 5 ml of LB medium without replacing the air with argon.

Construction of *P. aeruginosa* mutants. The plasmids used in this study are listed in Table 1. The PCR primers used in this study are listed in Table S1 in the supplemental material. pG19ccoN1 and pG19ccoN2 plasmids carrying deletion cassettes of *ccoN1* and *ccoN2* were constructed based on a previously described method (13). The pG19II-derived plasmids (pG19ccoN1 and pG19ccoN2) were transferred into *P. aeruginosa* PAO1 wild-type (WT) and Δ *ccoN1* strains by conjugation with the mobilizer *E. coli* S17-1 (14), followed by homologous recombination as previously described (13). Target gene deletions were confirmed by PCR analysis. In addition, to construct the Δ *ccoN1* Δ *ccoN2* Δ *nir* mutant, which is devoid of NIR and does not produce NO, pG19nir (15) was transferred into the Δ *ccoN1* Δ *ccoN2* strain by conjugation with *E. coli* S17-1, followed by homologous recombination.

C23O specific activity assay. The *ccoN1* and *ccoN2* transcriptional fusion plasmids were constructed by cloning the promoter regions of *ccoN1* and *ccoN2* (4, 5) into the pMEX9 reporter plasmid upstream of *xylE*. These plasmids were introduced into the *P. aeruginosa* PAO1 WT (16). The specific activities of catechol 2,3-dioxygenase (C23O) (the *xylE* gene product) in the strains carrying reporter plasmids were determined by monitoring the absorbance at 375 nm (A₃₇₅) by following a method described previously (13).

DIC and bright-field observation. For differential interference contrast (DIC) observation of cells in planktonic shaking cultures, 5- μ l quantities of the cultures were mounted onto micro-cover glasses (VWR Inter-

national, USA) by following a method described previously (17). For the bright-field observation of cells in biofilms, biofilms formed in 6-well plates were washed with phosphate-buffered saline (PBS). After scraping of the biofilms with PBS, 10- μ l quantities of the suspensions were mounted onto micro-cover glasses. A Carl Zeiss laser scanning microscope (LSM 710) equipped with a 63 \times /1.40 numerical aperture Plan-Apochromat objective (Carl Zeiss, Oberkochen, Germany) was used to acquire DIC and bright-field images. The images were captured using an AxioCam MRm digital camera and analyzed with the AxioVision software (version 4.8; Carl Zeiss).

Denitrification activity assay. Denitrification activities were measured by analytical methods described previously (9, 12). NO₃⁻ concentrations were determined by the brucine-sulfanilic acid method (18). NO₂⁻ concentrations were determined by the sulfanilamide-naphthylethylene diamine method (18). N₂O and N₂ concentrations were measured with a gas chromatograph (model GC-8AIT; Shimadzu).

DPA assay. Quantification of the cellular levels of deoxynucleoside triphosphates (dNTPs) in cell extracts was performed based on a previously described method (19). Anoxic incubation of *P. aeruginosa* was performed using butyl-rubber-sealed 500-ml Erlenmeyer flasks containing 80 ml of LBN medium at 37°C with shaking at 200 rpm. Anoxically grown cells were washed in 100 mM potassium phosphate buffer (pH 7.5) and sonicated. Lysed cell extracts containing equal amounts of protein were mixed with diphenylamine (DPA) reagent, consisting of 2 g of DPA (Wako, Osaka, Japan) dissolved in 100 ml of pure acetic acid and 2.75 ml of concentrated sulfuric acid. After incubation at 37°C for 4 h, the A₅₉₅ was measured.

NO detection. Cellular NO levels were measured based on a previously described method (20) with a NO detection reagent, diaminofluorescein-2 diacetate (DAF-2 DA) (Sekisui Medical, Tokyo, Japan). One-milliliter quantities of planktonic shaking cultures grown under anoxic conditions were incubated with 10 μ M DAF-2 DA. After incubation at 37°C for 1 h and washing with PBS, the green fluorescence of a reaction product, DAF-2 T, was measured using a Varioskan flash fluorometer (Thermo Scientific, Waltham, MA) at an excitation wavelength of 495 nm

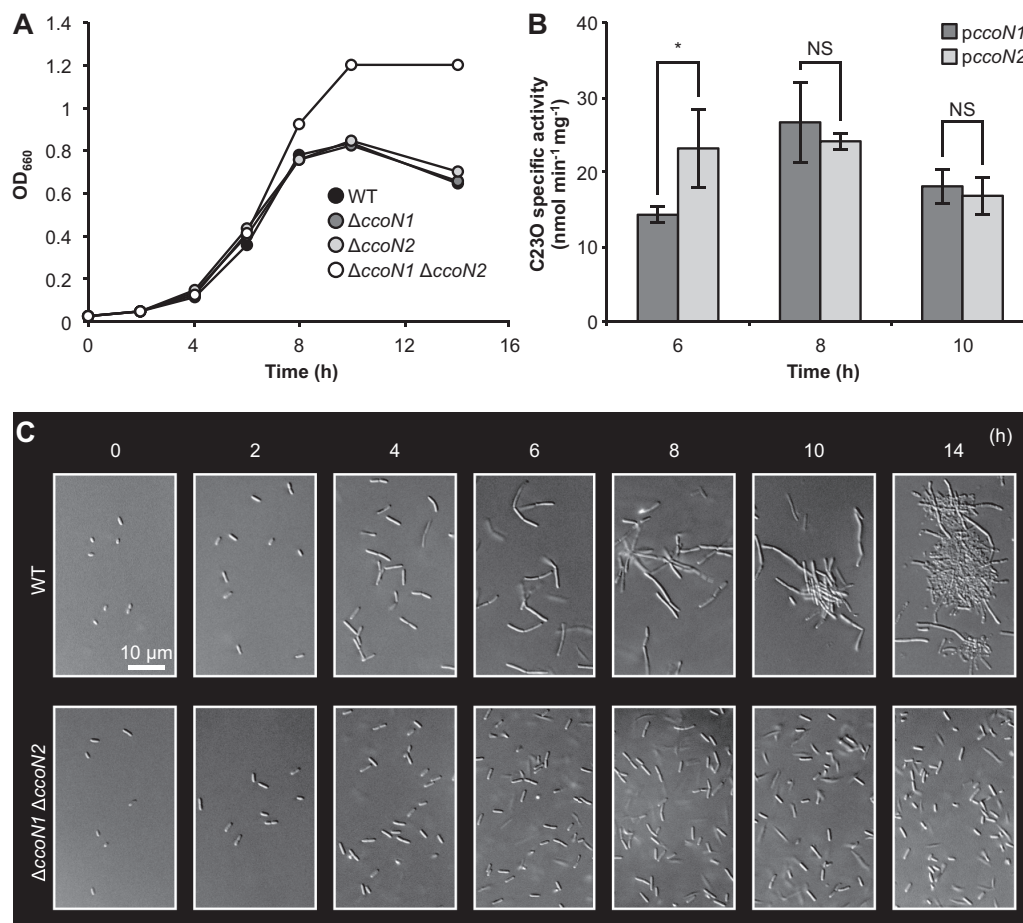


FIG 1 Effect of *cbb*₃ oxidases on anaerobic growth and cell morphology. Anoxic incubation of *P. aeruginosa* was performed using Hungate tubes containing argon gas and LBN medium at 37°C with shaking at 200 rpm. (A) Time course of OD measurements. Three independent experiments were carried out, and representative data are shown. (B) Transcriptional activities of two *cbb*₃ oxidases. *ccoN* promoter-dependent C23O activities in cells possessing pMEXccoN1 or pMEXccoN2 were measured and normalized to the C23O activities in cells possessing pMEX9. The values represent the means \pm standard deviations from three independent experiments. NS, not significant. *, $P < 0.05$ (unpaired two-tailed Student's *t* test). (C) Time course of DIC images. Three independent experiments were carried out, and representative images are shown.

and an emission wavelength of 515 nm. The fluorescent intensity was normalized to OD₆₆₀.

Biofilm formation assay. Overnight cultures of *P. aeruginosa* grown under oxic conditions were diluted to an OD₆₆₀ of 0.1 in LBN medium. Aliquots (100 μ l) of the cultures were inoculated into 900 μ l of LBN medium in 24-well polystyrene plates to achieve an OD₆₆₀ of 0.01. The plates were incubated at 37°C under anoxic conditions. For anoxic incubation, an AnaeroPack system (Mitsubishi Gas Chemical, Tokyo, Japan) was used (17). After incubation, quantification of biofilms was performed with the crystal violet staining method, as previously described (17, 21).

Biofilm visualization with COCRM. For biofilm visualization, 6-well polystyrene plates were used. Overnight cultures of *P. aeruginosa* grown under oxic conditions were diluted to an OD₆₆₀ of 0.5 in LBN medium. Aliquots (0.1 ml) of the cultures were inoculated into 4.9 ml of LBN medium in 6-well plates to achieve an OD₆₆₀ of 0.01. The plates were anoxically incubated at 37°C with an AnaeroPack system. After incubation for 24 h, biofilms that had formed at the bottom of the 6-well plates were washed with PBS, and the structures were visualized with continuous-optimizing confocal reflection microscopy (COCRM) (22, 23). A Carl Zeiss LSM 5 PASCAL equipped with a 63 \times /0.9 numerical aperture W N-Achroplan water immersion objective was used to acquire COCRM images. Biofilms were illuminated with a 514-nm argon laser, and the

reflected light was collected. COCRM images were analyzed with the LSM 5 PASCAL software (version 3.5; Carl Zeiss).

LIVE/DEAD bacterial viability assay. The bacterial viability in planktonic shaking cultures was determined by using a BacLight LIVE/DEAD bacterial viability staining kit (Molecular Probes, Eugene, OR), by following a previously described method (24). A Carl Zeiss LSM 5 PASCAL equipped with a 63 \times /1.4 numerical aperture Plan-Apochromat objective was used to acquire LIVE/DEAD images. Live SYTO9-stained cells and dead propidium iodide-stained cells were excited with an argon laser and a helium neon laser, respectively, and detected with a 560-nm long-pass filter and a 505- to 530-nm band-pass filter, respectively. LIVE/DEAD images were analyzed with the LSM 5 PASCAL software (version 3.5; Carl Zeiss).

RESULTS

***cbb*₃ oxidases alter cell morphology under anaerobic denitrifying growth.** To investigate the effect of two *cbb*₃ oxidases on cell growth, *P. aeruginosa* PAO1 WT and $\Delta ccoN$ deletion mutants that are deficient in *cbb*₃-1 and/or *cbb*₃-2 oxidases were incubated oxicallly in LB medium or anoxically in LBN medium. Under anoxic denitrifying planktonic conditions, the $\Delta ccoN1 \Delta ccoN2$ mutant

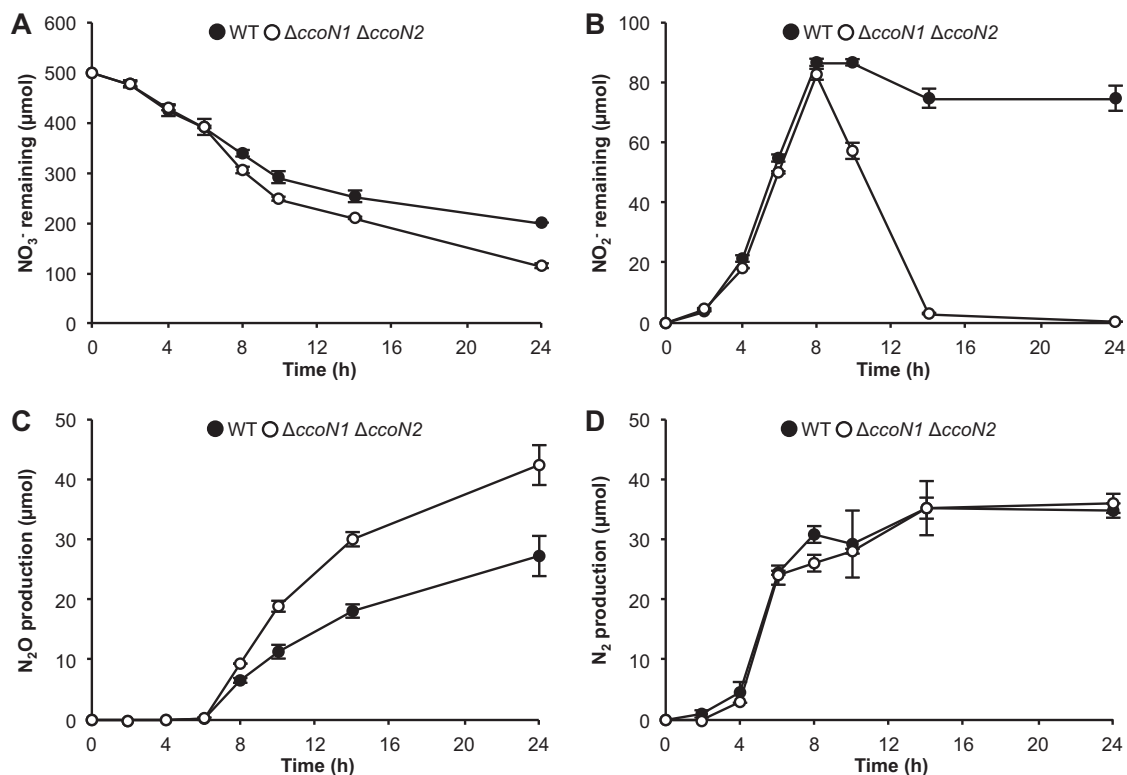


FIG 2 Effect of *cbb₃* oxidases on the denitrification process. The amounts of NO_3^- remaining (A), NO_2^- remaining (B), N_2O production (C), and N_2 production (D) were measured in planktonic shaking cultures. The values represent the means \pm standard deviations from three independent experiments.

grew better than the WT (Fig. 1A). Growth of ΔccoN single mutants was similar to that of the WT. The CFU value of the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant was higher than those of the WT and the ΔccoN single mutants at 14 h (see Fig. S2 in the supplemental material). These results show that *cbb₃*-1 and *cbb₃*-2 oxidases alter anaerobic denitrifying growth. *cbb₃*-1 subunits of *P. aeruginosa* have a high degree of amino acid similarity to *cbb₃*-2 subunits (4). Therefore, two *cbb₃* oxidases may complement each other under anoxic denitrifying conditions. On the other hand, aerobic planktonic growth of ΔccoN1 and $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutants was slightly delayed compared with that of the WT and the ΔccoN2 mutant (see Fig. S3A). In addition, the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant showed less growth than the WT under hypoxic conditions in the absence of nitrate (see Fig. S4), which was consistent with a previous report (25).

Kawakami et al. examined the expression levels of *cbb₃* oxidases under different oxygen concentrations (5). To monitor whether *cbb₃* oxidases were expressed under our experimental conditions, promoter fusion plasmids with the *xylE* gene fused with promoter regions of the *ccoN1* and *ccoN2* genes were used. The specific activities of C23O, the *xylE* gene product, were determined by a previously described method (13). As expected, promoter expression of *cbb₃*-1 and *cbb₃*-2 oxidases in the *P. aeruginosa* PAO1 WT was confirmed during the logarithmic to stationary phase (Fig. 1B), suggesting that these respiratory enzymes have a physiological role under anoxic denitrifying conditions. In a study by Yoon et al., it was demonstrated that *P. aeruginosa* PAO1 elongates and aggregates in anoxic planktonic shaking cultures (26). Similar phenomena were observed in the WT by DIC observation. In contrast, the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant did not elongate under anoxic

denitrifying conditions (Fig. 1C). Both strains were rod shaped under oxic conditions (see Fig. S3B). These results show that *cbb₃* oxidases alter cell morphology under anaerobic denitrifying growth.

***cbb₃* oxidases alter the denitrification process.** Under anoxic conditions where NO_3^- is present, *P. aeruginosa* can grow through denitrification, in which NO_3^- is reduced subsequently to NO_2^- , NO, N_2O , and, finally, to N_2 (3). Cell elongation under anoxic conditions is attributed to the accumulation of NO that is produced during such a process (26). Our data showed that *cbb₃* oxidases affect cell morphology; hence, the effect of *cbb₃* oxidases on the denitrification process was investigated. As shown in Fig. 2A and B, the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant reduced more NO_3^- and NO_2^- than the WT. N_2O production was higher in the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant than in the WT (Fig. 2C). N_2 production of the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant was similar to that of the WT (Fig. 2D). These results show that *cbb₃* oxidases alter the denitrification process. As shown by our data, the amounts of NO_2^- and N_2O were most affected by the *cbb₃* oxidases, suggesting that *cbb₃* oxidases alter the denitrification process in between the reduction of NO_2^- to N_2O .

***cbb₃* oxidases alter cell morphology through NO.** Because NO accumulation induces cell elongation (26), it can be hypothesized that *cbb₃* oxidases affect NO accumulation, which leads to cell elongation. To test this hypothesis, NIR mutants that do not produce NO were used. Compared with the growth difference between the WT and the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant, Δnir and $\Delta\text{ccoN1 } \Delta\text{ccoN2 } \Delta\text{nir}$ mutants had similar but not identical growth (Fig. 1A and Fig. 3A and B; see also Fig. 4B), suggesting that NO is one

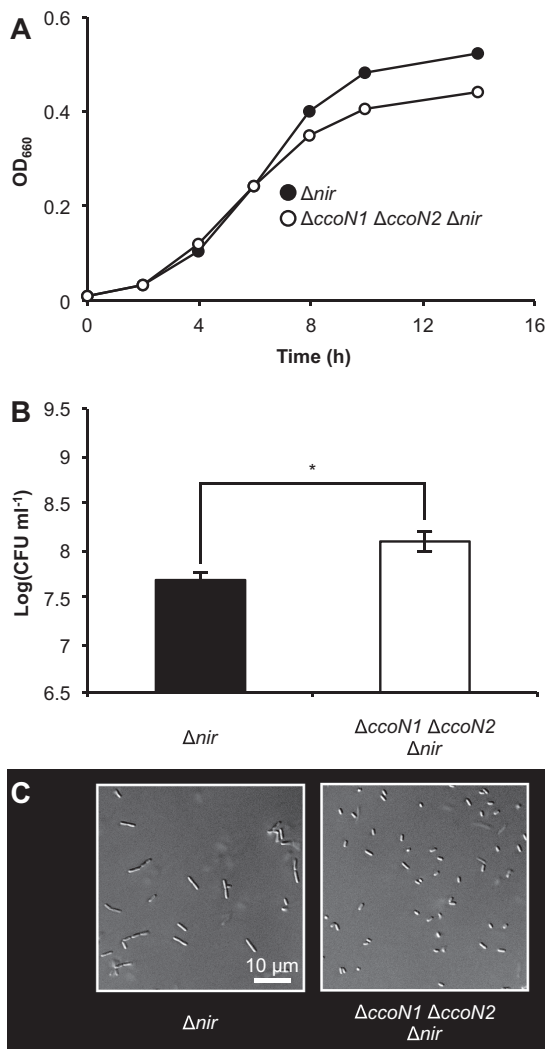


FIG 3 Anaerobic growth and cell morphology of NIR mutants. (A) Time course of OD measurements. Three independent experiments were carried out, and representative data are shown. (B) CFU at 14 h. The values represent the means \pm standard deviations from three independent experiments. *, $P < 0.01$ (unpaired two-tailed Student's *t* test). (C) DIC images of NIR mutants at 14 h. Three independent experiments were carried out, and representative images are shown.

of the main causes of the growth difference. Consistent with other reports (26), the Δnir mutant did not elongate as the WT and was rod shaped, similar to the $\Delta ccoN1 \Delta ccoN2$ mutant (Fig. 1C and 3C). When planktonic shaking cultures were observed by LIVE/DEAD staining, the WT culture was found to comprise dead or membrane-damaged cells, while the NIR mutant culture comprised intact cells (see Fig. S5 in the supplemental material). This result was consistent with previous observations (27, 28). The $\Delta ccoN1 \Delta ccoN2$ mutant culture comprised intact cells, similar to NIR mutants. These results support the hypothesis that *cbb*₃ oxidases affect NO accumulation during denitrification.

NO derived from denitrification inhibits DNA synthesis by ribonucleotide reductase (RNR), especially class II-type RNR (RNR II), during anaerobic growth of *P. aeruginosa* PAO1 (19). Because RNR catalyzes the formation of deoxyribonucleotides from ribonucleotides (29), cellular dNTP levels in the WT and the

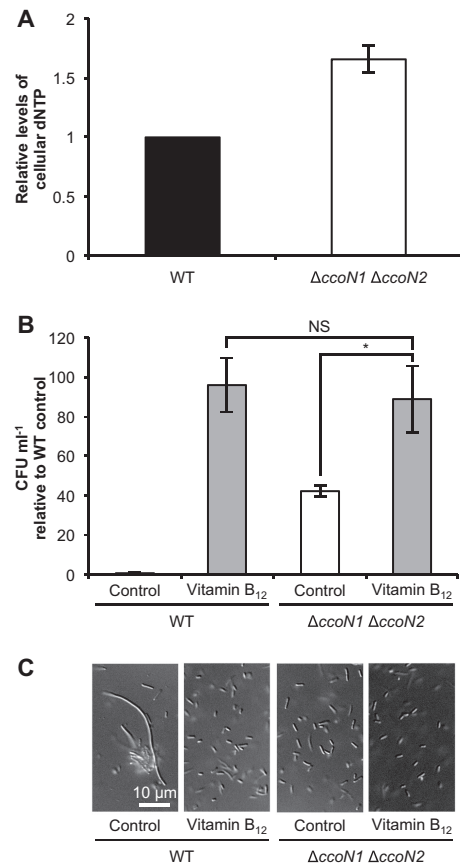


FIG 4 Effect of *cbb*₃ oxidases on DNA synthesis. (A) Relative cellular dNTP levels at 14 h as determined by a DPA assay. Anoxic incubation of *P. aeruginosa* was performed using Erlenmeyer flasks containing argon gas and LBN medium at 37°C with shaking at 200 rpm. Cell extracts containing equal amounts of protein were used in this assay. The values represent the means \pm standard deviations from three independent experiments. (B) Relative CFU at 24 h in LBN medium supplemented with 100 nM vitamin B₁₂. The values represent the means \pm standard deviations from three independent experiments. NS, not significant. *, $P < 0.05$ (unpaired two-tailed Student's *t* test). (C) DIC images at 24 h. Three independent experiments were carried out, and representative images are shown.

$\Delta ccoN1 \Delta ccoN2$ mutant were quantified by a DPA assay. The dNTP level of the $\Delta ccoN1 \Delta ccoN2$ mutant was higher than that of the WT (Fig. 4A), suggesting that *cbb*₃ oxidases inhibit RNR II activity, presumably through NO. Vitamin B₁₂, a coenzyme of RNR II, has been reported to restore defective anaerobic growth caused by NO (19). In the absence of vitamin B₁₂, the $\Delta ccoN1 \Delta ccoN2$ mutant grew better than the WT. When supplemented with vitamin B₁₂, growth of the $\Delta ccoN1 \Delta ccoN2$ mutant was similar to that of the WT (Fig. 4B). Furthermore, cell morphology of the $\Delta ccoN1 \Delta ccoN2$ mutant was similar to that of the WT in the presence of vitamin B₁₂ (Fig. 4C). Growth of the $\Delta ccoN1 \Delta ccoN2$ mutant supplemented with vitamin B₁₂ was slightly higher and the cell length was shorter than for the control, suggesting that NO also influences anaerobic growth and cell morphology of the $\Delta ccoN1 \Delta ccoN2$ mutant. Taken together, these results indicate that *cbb*₃ oxidases promote cell elongation under anoxic denitrifying conditions by affecting NO accumulation.

***cbb*₃ oxidases alter the cellular NO level.** To provide further evidence that *cbb*₃ oxidases induce NO accumulation, cellular NO

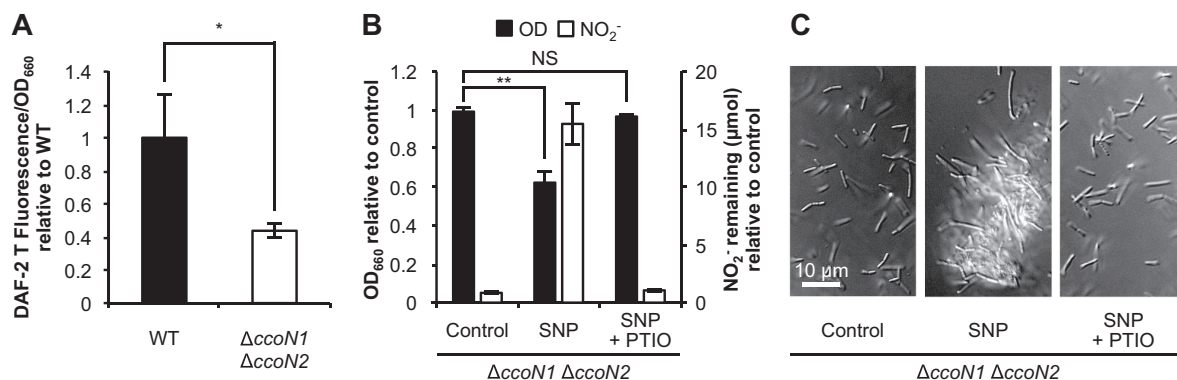


FIG 5 Effect of *cbb₃* oxidases on the cellular NO level. (A) Relative cellular NO levels at 6 h as determined by a NO detection reagent, DAF-2 DA. The values represent the means \pm standard deviations from three independent experiments. *, $P < 0.05$ (unpaired two-tailed Student's *t* test). (B) Relative ODs and the amounts of NO_2^- remaining for the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant at 14 h in LBN medium supplemented with $1 \mu\text{M}$ NO donor SNP and/or 2 mM NO scavenger PTIO. The values represent the means \pm standard deviations from three independent experiments. NS, not significant. **, $P < 0.01$ (unpaired two-tailed Student's *t* test). (C) DIC images at 14 h. Three independent experiments were carried out, and representative images are shown.

levels of the WT and the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant were measured with a NO detection reagent, DAF-2 DA. As expected, the NO levels of the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant were lower than for the WT (Fig. 5A). Furthermore, the WT phenotype was restored in the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant when a NO donor, sodium nitroprusside (SNP; Sigma-Aldrich, St. Louis, MO), was added in the culture. Addition of SNP resulted in low growth yield of the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant and induced the accumulation of NO_2^- , as observed in the WT (Fig. 1A and 2B), compared with those of the control (Fig. 5B). In addition, elongated cells and cell aggregates observed in the WT (Fig. 1C) were observed in the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant by the addition of SNP (Fig. 5C). The influence of SNP was diminished by the addition of a NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (PTIO; Wako), confirming that NO but not iron or cyanide released from SNP affected the cell physiology of the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant. These results show that *cbb₃* oxidases alter the cellular NO level during the denitrification process.

***cbb₃* oxidases promote the formation of biofilms composed of elongated cells.** As shown in Fig. 1C, *cbb₃* oxidases promote bacterial aggregation under anoxic planktonic conditions. Cell aggregation often shares common mechanisms with biofilm formation (30, 31). Therefore, *cbb₃* oxidases may also influence bacterial biofilm formation under anoxic static conditions. The insets in Fig. 6A show crystal violet-stained biofilms formed at the bottom of 24-well plates. From the initial attachment stage (0 to 12 h), the amount of biofilm formed by the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant was less than that formed by the WT (Fig. 6A). At 24 h, the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant formed half as much biofilm as the WT. Biofilm formation of ΔccoN single mutants was similar to that of the WT (see Fig. S6 in the supplemental material). These results show that *cbb₃*-1 and *cbb₃*-2 oxidases alter anaerobic biofilm formation. As shown in Fig. S7A in the supplemental material, the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant reduced NO_3^- earlier than the WT under this condition. NO_2^- was less accumulated in the culture of the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant than in that of the WT (see Fig. S7B), supporting our idea that *cbb₃* oxidases alter anaerobic biofilm formation through denitrification. A previous study demonstrated that cell elongation promoted by NO is required for robust biofilm formation of *P. aeruginosa* PAO1 under anoxic denitrifying conditions (26). In

contrast with the difference in biofilm formation between the WT and the $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ mutant, Δnir and $\Delta\text{ccoN1 } \Delta\text{ccoN2 } \Delta\text{nir}$ mutants, which do not produce NO, formed similar amounts of biofilms (Fig. 6A and B), suggesting that NO is one of the main factors responsible for the difference in biofilm formation. Cell morphological changes in biofilms were confirmed by the bright-field observation of cells scraped from biofilms and the observation of biofilm structures using COCRM (22, 23). The WT biofilms were composed of elongated cells, while $\Delta\text{ccoN1 } \Delta\text{ccoN2}$ and NIR mutant biofilms were composed of rod-shaped cells (Fig. 6C). These results show that *cbb₃* oxidases promote mature biofilm formation via cell elongation under anoxic denitrifying conditions. Because of NO accumulation by the *cbb₃* oxidases (Fig. 5A), cell elongation and the formation of biofilms composed of elongated cells are triggered.

DISCUSSION

In this study, we uncovered a novel role for *cbb₃* oxidases in anoxic denitrifying environments in *P. aeruginosa* PAO1. It was shown that *cbb₃* oxidases induce NO accumulation during the denitrification process and promote the maturation of the anaerobic biofilm structure via cell elongation.

Impact of *cbb₃* oxidases on cell behaviors under anoxic conditions. *cbb₃* oxidases of *P. aeruginosa* are more highly expressed under anoxic denitrifying conditions (oxygen concentration of 0%) than under oxic conditions (5). Similarly, Rosenbaum et al. demonstrated transcriptional expression of *cbb₃* oxidase during biofilm formation of *Shewanella oneidensis* MR-1 under anoxic conditions (20% CO_2 –80% N_2) when an electrode was used as a terminal electron acceptor; however, the role of *cbb₃* oxidase is not well understood (32). In *P. aeruginosa*, under oxic conditions, *cbb₃* oxidases support energy conservation by catalyzing oxygen reduction in aerobic respiration. Consistent with its role in conserving energy under low-oxygen conditions, the deletion of *cbb₃* oxidases resulted in less biofilm formation in the oxic flow cell chamber than for the WT, where oxygen becomes limited (25). Our current study was carried out under anoxic conditions, and *cbb₃* oxidases were shown to promote biofilm formation (Fig. 6A). Rather than through energy conservation, we hypothesize that *cbb₃* oxidases affect biofilm formation through NO stress. Growth

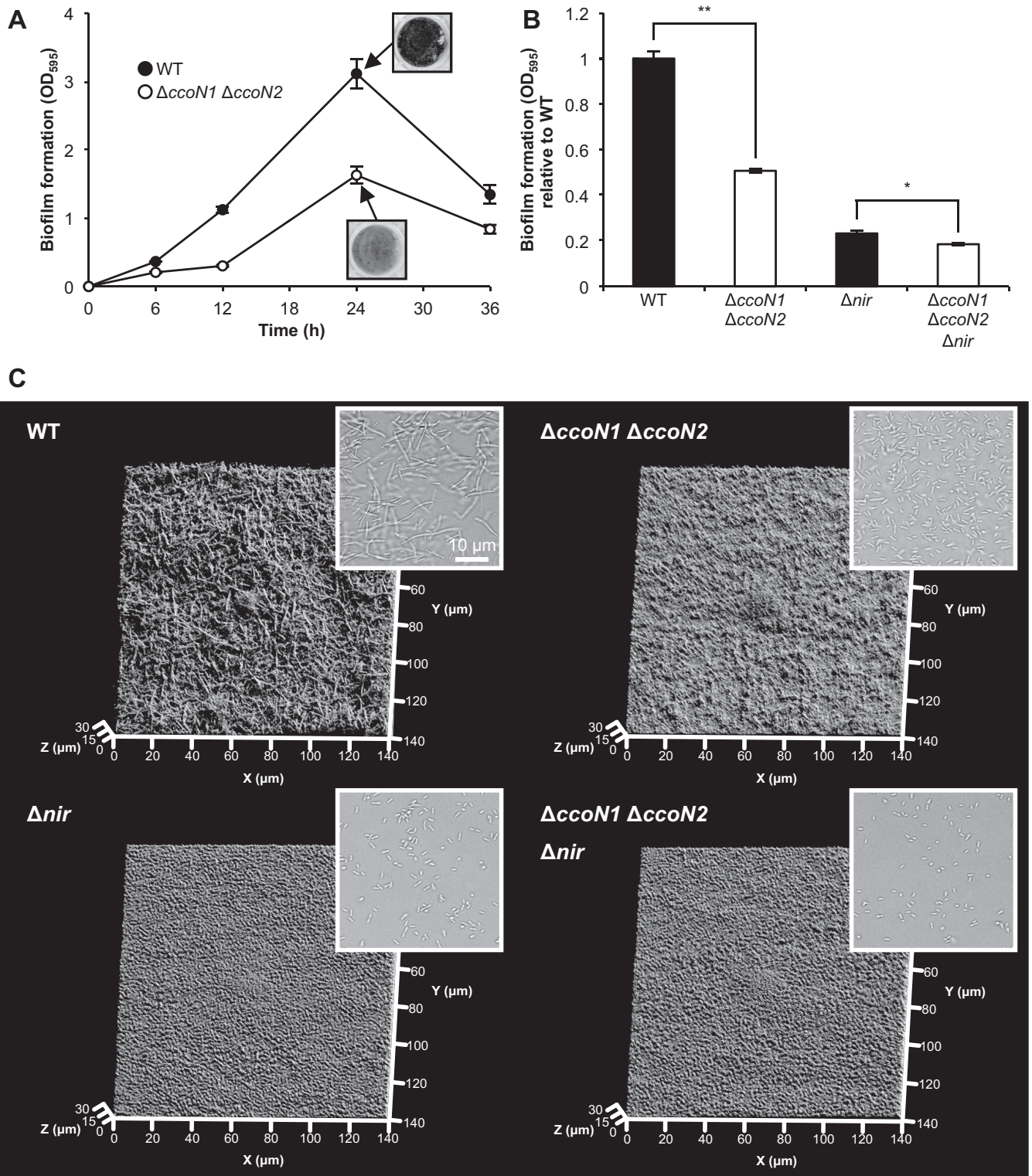


FIG 6 Effect of *cbb*₃ oxidases on biofilm formation. Anoxic incubation of *P. aeruginosa* was performed using 24-well (A and B) or 6-well (C) plates containing LBN medium incubated at 37°C. An AnaeroPack system was used for anoxic static incubation. (A) Time course of the amounts of biofilm formation. The values represent the means \pm standard deviations from three independent experiments. The insets show crystal violet-stained biofilms formed at the bottom of 24-well plates. (B) Relative amounts of biofilm formation of NIR mutants at 24 h. The values represent the means \pm standard deviations from three independent experiments. *, $P < 0.01$; **, $P < 0.0001$ (unpaired two-tailed Student's *t* test). (C) Three-dimensional images of biofilm structures at 24 h visualized by COCRM. Each projection shows fields of 140 by 140 μm (*x-y*), as indicated. The insets show the bright-field images of cells scraped from biofilms. Three independent experiments were carried out, and representative images are shown.

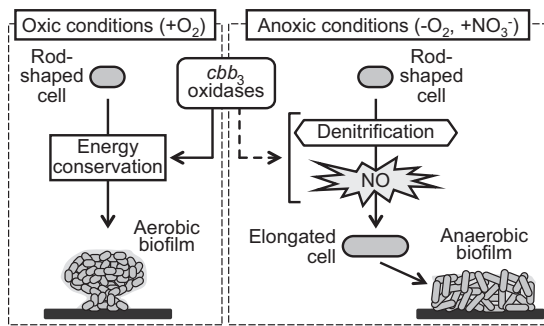


FIG 7 Proposed model depicting how the roles of *cbb₃* oxidases differ depending on the environment. Under oxic conditions, *cbb₃* oxidases support energy conservation through oxygen reduction, resulting in aerobic biofilm formation. On the other hand, under anoxic denitrifying conditions, *cbb₃* oxidases lead to NO accumulation during the denitrification process. NO promotes cell elongation and subsequent anaerobic biofilm formation. It is unclear how *cbb₃* oxidases induce NO accumulation.

of the $\Delta ccoN1 \Delta ccoN2$ mutant was similar to that of the WT in the presence of vitamin B₁₂, which alleviates NO stress (Fig. 4B). Moreover, *cbb₃* oxidases led to the accumulation of NO (Fig. 5A). These results support our hypothesis that NO stress rather than energy conservation is a key factor that determines the effect of *cbb₃* oxidases under anoxic conditions. Consistent with this idea, Yoon et al. demonstrated that NO stress derived from denitrification induces cell elongation, leading to anaerobic biofilm formation (26). Taken together, our data show that *cbb₃* oxidases have different roles depending on the environment (Fig. 7).

NO is involved in regulating several phenotypes related to cell-cell interactions and social behaviors in *P. aeruginosa*. For instance, NO can promote membrane vesicle (MV) production and type III secretion system (T3SS) expression, which are involved in host infection (15, 33). NO not only promotes biofilm formation under anoxic conditions (26) but also functions as a signal to induce biofilm dispersal under certain conditions (28). Although NO plays important roles in cell physiology, how NO accumulation is controlled is poorly understood. QS has been indicated to balance the activities of NIR and NOR enzymes, thus controlling NO accumulation, while the precise mechanism is unknown (8, 9, 27). Our results show that *cbb₃* oxidases are also involved in the control of this important molecule and further implying the impact of the *cbb₃* oxidases in regulating cell behaviors.

In *P. aeruginosa* PAO1, cell elongation through NO stress is required for mature biofilm formation under anoxic conditions (26). Under anoxic environments, specific mat-like biofilm structures composed of elongated cells are formed, which are different from the mushroom-like biofilm structures composed of rod-shaped cells that form under oxic environments. It is suggested that cell elongation and mature biofilm formation can provide an advantage under stressful environmental conditions (10, 11). However, some clinical isolates of *P. aeruginosa* remain rod shaped and form mature biofilms under anoxic conditions (17). This result indicates the presence of unknown factors leading to mature biofilm formation in these clinical isolates. It will be interesting to determine whether *cbb₃* oxidases are involved in the variation among strains under anoxic conditions.

Although our data show that NO stress is the main cause for

cell elongation, the cells of the $\Delta ccoN1 \Delta ccoN2 \Delta nir$ mutant were shorter than those of the Δnir mutant under planktonic conditions (Fig. 3C), suggesting that other mechanisms that affect cell morphology may exist. Under static conditions, the cells of the $\Delta ccoN1 \Delta ccoN2 \Delta nir$ mutant were shorter than those of the Δnir mutant, and biofilm formation of the $\Delta ccoN1 \Delta ccoN2 \Delta nir$ mutant was decreased compared with that of the Δnir mutant (Fig. 6B and C). Thus, cell morphological changes affect anaerobic biofilm formation via unknown mechanisms. It has been reported that the mutation or overexpression of the terminal oxidase CIO results in a cell division defect in *P. aeruginosa* (34). *cbb₃* oxidases may also affect cell morphology via a common unknown mechanism under anoxic conditions.

Conclusions. Collectively, our data show that *cbb₃* oxidases of *P. aeruginosa* PAO1 lead to NO accumulation during the denitrification process and promote biofilm formation via cell elongation in anoxic denitrifying environments. It is unique that *cbb₃* oxidases are strongly involved in biofilm formation in anoxic environments where oxygen-dependent respiratory activity is not dominant. A few studies have reported an additional role for bacterial respiratory enzymes. For example, mutations in the *cyo* operon encoding cytochrome *o* ubiquinol oxidase influence carbon catabolite repression of phenol degradation by *Pseudomonas putida* (35). Our data reveal a new aspect of *cbb₃* oxidases that depends on environmental conditions and further imply the multifunctionality of bacterial respiratory enzymes.

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