An Aerobic Exercise: Defining the Roles of *Pseudomonas aeruginosa* Terminal Oxidases

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ABSTRACT: The opportunistic pathogen *Pseudomonas aeruginosa* encodes a large and diverse complement of aerobic terminal oxidases, which is thought to contribute to its ability to thrive in settings with low oxygen availability. In this issue, Arai et al. present a thorough characterization of these five complexes, enabling a more detailed understanding of aerobic respiration in this organism.

Although the Earth’s atmosphere contains approximately 21% oxygen, bacteria in environments that are ostensibly aerobic still encounter zones where the concentration is much lower, due to the effects of oxygen solubility, low diffusibility, and consumption by neighboring cells. Many bacteria cope with this in part through the use of branched respiratory chains that can be modulated in response to changing conditions (1). Aerobic terminal oxidases, which catalyze electron transfer from the respiratory apparatus to oxygen, vary in their affinities and efficiencies (2). Five such enzymes have been identified in the opportunistic pathogen *Pseudomonas aeruginosa* (Fig. 1), and these are thought to contribute to its ability to thrive under hypoxia (1, 3-8). A diverse collection of aerobic terminal oxidases may be especially critical for an organism that has a proficiency for persisting in biofilms, which are characterized by the formation of steep oxygen gradients (9-11). In addition to contributing to *P. aeruginosa*’s growth under microaerobic conditions, some of these complexes have been implicated in its ability to cope with various stresses (6, 12). In this issue of the *Journal of Bacteriology*, Arai et al. conduct a comprehensive study of these complexes, probing their biochemical properties and contributions to aerobic growth (13). They describe a systematic investigation of the attributes of each enzyme, which clarified many aspects
of *P. aeruginosa*'s aerobic biology and revealed a new potential role for a particular high-affinity oxidase.

The *P. aeruginosa* aerobic terminal oxidases include enzymes that can use ubiquinol or cytochromes as electron donors, ones that have low or high affinities for O$_2$, and a cyanide-insensitive oxidase (CIO) that functions in the presence of this endogenously produced virulence factor (12). Aside from CIO, the four other aerobic terminal oxidases encoded by the *P. aeruginosa* genome are the bo$_3$ oxidase (Cyo), the aa$_3$ oxidase (Aa3), cbb$_3$ oxidase-1 (Cbb3-1), and cbb$_3$ oxidase-2 (Cbb3-2). Earlier studies predicted that Cyo and Aa3 (which is most similar to the mitochondrial terminal oxidase) would have low affinities for oxygen and that CIO and the Cbb3 enzymes would have high oxygen affinities (4-6, 14, 15). In batch cultures grown under typical conditions (i.e., in nutrient-rich media and atmospheric oxygen, with vigorous shaking), the low-affinity enzymes Cyo and/or Aa3 would thus be expected to play major roles during exponential growth, when oxygen is relatively abundant. The high-affinity enzymes Cbb3-1 and Cbb3-2 would be expected to function primarily in stationary phase, when oxygen is relatively scarce, and/or during growth in microaerobic conditions. Hypothetically, CIO would be particularly important in stationary phase, as it is the only terminal oxidase that functions in the presence of cyanide, which *P. aeruginosa* produces at high cell density (7, 12, 16).

Using gene expression and mutant analyses, previous studies have revised and clarified this working model of *P. aeruginosa* aerobic respiration. They have shown that the Aa3 oxidase, which plays a major role in aerobic respiration in other bacteria (14, 15), is expressed primarily under nutrient-limited conditions and is otherwise a minor
player under typical conditions (6). Rather, the Cbb3-1, Cbb3-2, and ClO oxidases play
major roles, and their importance is exaggerated when oxygen becomes limiting and
during growth in biofilms (4-6, 17). The two Cbb3 oxidases are very similar at the amino
acid level (with a similarity of 87% for the catalytic subunit in strain PAO1), but they are
differentially regulated: the Cbb3-1 complex is expressed constitutively, while the Cbb3-
2 complex is induced by oxygen limitation (4, 5). Critically, these studies also showed
that oxidase gene expression can be compensatory, such that loss of one or more
oxidases leads to induction of others (6).

To study the physiological roles of the *P. aeruginosa* aerobic terminal oxidases in
isolation, Arai et al. took the important step of generating combinatorial mutants, each
containing only one of these complexes, in strain PAO1. Growth experiments confirmed
the unique primary physiological roles of the Cbb3 complexes in this bacterium (4-6).
Under typical conditions, the strain containing the Cbb3-1 oxidase grew like the wild
type, while the strain containing the Cbb3-2 oxidase showed a defect earlier in the
growth experiment before “catching up” to the final wild-type density. This growth profile
is consistent with the prior observation that Cbb3-2 is induced specifically by oxygen
limitation (4); basal levels of expression could support initial growth before increased
oxygen consumption by the denser culture brings the oxygen level down and thereby
enhances expression. The growth phenotypes of the other individual single-oxidase
strains also reflected what was known about regulation of the *P. aeruginosa* terminal
oxidases (4, 6).

Prior work looking at expression of genes encoding putative c-type cytochromes
had provided clues as to which of these proteins might carry electrons from the
cytochrome bc1 complex to the Cbb3-type oxidases (6). Arai et al. used a genetic approach to determine that cytochrome c4, encoded by ORF PA5490 in strain PAO1, is the predominant mediator functioning at this step in the electron transport pathway under typical and microaerobic conditions. This finding fills in an important gap that was remaining in our model of the P. aeruginosa aerobic respiratory chain.

To assess their positions in the electron transport chain and oxygen affinities, Arai et al. conducted in vitro studies with membranes prepared from strains producing each of the individual oxidases. Oxygen consumption assays confirmed the electron donor (ubiquinone or cytochrome c) predictions for each complex (4), as well as the cyanide sensitivity of all of the oxidases but CIO. A battery of affinity assays also confirmed predictions for the relative affinities of the oxidases, with one exception: CIO, a cytochrome bd-type enzyme, was found to differ from the canonical Escherichia coli cytochrome bd (18) in that it showed a low affinity for oxygen. The physiological consequences of the low oxygen affinity of CIO were examined by expressing the P. aeruginosa cioAB genes in an E. coli mutant lacking native terminal oxidases. The growth of this strain was compared to a version of the same mutant expressing the E. coli cytochrome bd under the same promoter. Growth rates and yields for the two strains were similar under typical conditions, but the CIO-expressing strain showed a slower growth rate under microaerobic conditions, further supporting the conclusion that CIO functions optimally when oxygen availability is relatively high.

By generating P. aeruginosa mutants that each expressed only one terminal oxidase, Arai et al. were able to address outstanding questions regarding oxygen respiration and provide a more complete picture of the aerobic electron transport chain.
in this pathogen. A particularly intriguing feature of the *P. aeruginosa* system is that a high-affinity oxidase, Cbb3-1, functions as the major oxygen reductase in well-aerated cultures (Fig. 1). The authors point out that this is a unique trait, as most other bacteria regulate high-affinity oxidases such that they are expressed specifically under low-oxygen conditions, with low-affinity enzymes similar to Aa3 and/or Cyo acting as the major oxidase(s) under high-oxygen conditions (14, 15, 19). Even the closely related *Pseudomonas putida*, which also possess two Cbb3-type enzymes, employs Cyo as its major oxidase under typical conditions (19). Though the measured proton translocation efficiency (protons translocated across the membrane per oxygen atom consumed) values of the Cbb3-type oxidases were lower than that of the Aa3 oxidase, they were higher than the efficiency value of CIO and comparable to that of the Cyo oxidase. Cbb3-1's critical role during exponential growth with relatively high oxygen availability raises the question of whether this enzyme contributes capabilities beyond oxygen reduction in typical *P. aeruginosa* cultures. The Cbb3 oxidases differ from other heme-copper oxidases in their subunit and binuclear active site composition exhibiting structural similarities to nitric oxide reductases (NORs) (20, 21). Cbb3 oxidase purified from *P. stutzeri* has been shown to have NOR activity (22), and *P. aeruginosa*'s Cbb3 oxidases have been implicated in anaerobic denitrification and NO-mediated effects on biofilm formation (17). In diverse organisms, effects of the Cbb3 oxidases on the expression of other metabolic genes and on biomineralization have suggested that they may be involved in sensing and responding to changes in redox homeostasis (4, 23, 24). Finally, results from the current study and others suggest that modulation of the *P. aeruginosa* respiratory chain is strongly influenced by environmental stresses, and the
different oxidases provide activity under distinct, specialized conditions (6, 7, 16). Aa3 is expressed during nutrient starvation, Cyo is expressed under iron starvation and in the presence of a NO-generating reagent, and CIO is induced by copper starvation and cyanide (6). As Arai et al. note, utilization of Cbb3-1 during exponential growth may provide protection from reactive oxygen species or another unknown source of stress.

Further investigation into the ecophysiology of P. aeruginosa biofilm formation and infection may uncover the significance of Cbb3 activity for this bacterium, which utilizes oxygen preferentially and yet dominates in environments where it is in short supply.

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We apologize to authors whose work could not be cited due to space limitations.

REFERENCES


Figure legend: The P. aeruginosa membrane-bound electron transport chain can employ five different oxygen reductases. Ubiquinone (Ub) is reduced by a dehydrogenase (not shown) and acts as the electron donor for the cytochrome bc1 complex, Cyo, or ClO. The cytochrome bc1 complex reduces a c-type cytochrome, which then acts as the electron donor for Aa3, Cbb3-1, or Cbb3-2. Heme-copper oxidases are represented by pink shapes. Oxidases that support growth better under microaerobic conditions are shaded yellow, while those that support growth better under typical conditions are shaded blue. These roles are influenced by intrinsic chemical...
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