Adaptation of aerobically growing Pseudomonas aeruginosa to copper starvation

FRANGIPANI, Emanuela, et al.

Abstract
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Reference

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Adaptation of Aerobically Growing *Pseudomonas aeruginosa* to Copper Starvation

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Restricted bioavailability of copper in certain environments can interfere with cellular respiration because copper is an essential cofactor of most terminal oxidases. The global response of the metabolically versatile bacterium and opportunistic pathogen *Pseudomonas aeruginosa* to copper limitation was assessed under aerobic conditions. Expression of *cioAB* (encoding an alternative, copper-independent, cyanide-resistant ubiquinol oxidase) was upregulated, whereas numerous iron uptake functions (including the siderophores pyoverdine and pyochelin) were expressed at reduced levels, presumably reflecting a lower demand for iron by respiratory enzymes. Wild-type *P. aeruginosa* was able to grow aerobically in a defined glucose medium depleted of copper, whereas a *cioAB* mutant did not grow. Thus, *P. aeruginosa* relies on the CioAB enzyme to cope with severe copper deprivation. A quadruple *cya cco1 cco2 cox* mutant, which was deleted for all known heme-copper terminal oxidases of *P. aeruginosa*, grew aerobically, albeit more slowly than did the wild type, indicating that the CioAB enzyme is capable of energy conservation. However, the expression of a *cioA-lacZ* fusion was less dependent on the copper status in the quadruple mutant than in the wild type, suggesting that copper availability might affect *cioAB* expression indirectly, via the function of the heme-copper oxidases.

Copper is an essential micronutrient for most living organisms, as it participates in electron transport and in many biologically important redox reactions. The ability of copper to cycle between an oxidized Cu(II) state and a less stable reduced Cu(I) state makes it an important catalytic cofactor of cytochrome oxidases, the terminal enzymes in cellular respiration, and of other oxidases utilizing dioxygen (18). However, copper can become highly cytotoxic if allowed to accumulate in excess of cellular needs, as it is involved in the production of reactive oxygen species, including hydroxyl radicals (20). Therefore, both prokaryotes and eukaryotes must tightly regulate copper homeostasis (44).

Bacteria have evolved different strategies to maintain the intracellular copper concentration at a low level and within a narrow range (8). Two major types of mechanisms that prevent a copper overload in gram-negative bacteria have been described. One type involves periplasmic multicopper oxidases and copper-sequestering proteins, which are expressed under the control of two-component systems sensing periplasmic copper and copper-sequestering proteins, which are expressed under the control of two-component systems sensing periplasmic copper. Copper is an essential cofactor of most terminal oxidases. The global response of the metabolically versatile bacterium and opportunistic pathogen *Pseudomonas aeruginosa* to copper limitation was assessed under aerobic conditions. Expression of *cioAB* (encoding an alternative, copper-independent, cyanide-resistant ubiquinol oxidase) was upregulated, whereas numerous iron uptake functions (including the siderophores pyoverdine and pyochelin) were expressed at reduced levels, presumably reflecting a lower demand for iron by respiratory enzymes. Wild-type *P. aeruginosa* was able to grow aerobically in a defined glucose medium depleted of copper, whereas a *cioAB* mutant did not grow. Thus, *P. aeruginosa* relies on the CioAB enzyme to cope with severe copper deprivation. A quadruple *cya cco1 cco2 cox* mutant, which was deleted for all known heme-copper terminal oxidases of *P. aeruginosa*, grew aerobically, albeit more slowly than did the wild type, indicating that the CioAB enzyme is capable of energy conservation. However, the expression of a *cioA-lacZ* fusion was less dependent on the copper status in the quadruple mutant than in the wild type, suggesting that copper availability might affect *cioAB* expression indirectly, via the function of the heme-copper oxidases.

Whereas many studies have focused on how bacterial cells avoid copper toxicity, less is known about how microorganisms react to and cope with copper deficiency. In aqueous solutions under oxic conditions, copper is present in its cupric Cu(II) form. Above pH 7.4, Cu(II) can form poorly soluble carbonates and hydroxides (27). In biological fluids, copper is mostly bound to organic molecules. In human serum, the concentration of free Cu(II) is estimated to be about $10^{-13}$ M, mainly due to the complexion of copper with plasma proteins such as albumin, ceruloplasmin, and transcuprein (24, 32). The fact that copper can be poorly bioavailable raises the question of how environmental and pathogenic microorganisms adapt to copper limitation. There are scattered reports in the literature on this issue. For instance, in the marine bacterium *Pseudomonas perfectomarinina* (now called *Pseudomonas stutzeri*), a lack of copper interferes with the last step of denitrification, i.e., the reduction of nitrous oxide to dinitrogen, which is catalyzed by a copper-containing enzyme (33). In the cyanobacterium *Synechocystis* sp., copper deprivation causes an arrest of respiratory metabolism because cytochrome c oxidase fails to function, whereas photoautotrophic growth remains possible (17). Some methane-oxidizing bacteria can scavenge copper ions by producing specific chalkophores (copper chelators); chalkophores are akin to siderophores, which are iron chelators and provide iron to iron-starved cells (29). In the yeast *Saccharomyces cerevisiae*, copper starvation results in the downregulation of respiratory functions and reveals a link between copper and iron metabolism (50).
We have begun to study the adaptation of *P. aeruginosa* to copper limitation. *P. aeruginosa* is a widely occurring environmental bacterium and a pathogen in compromised hosts (25, 41); as such, it is likely to encounter situations of limited copper availability. The ability of *P. aeruginosa* to cause disease is based not only on its capacity to produce a large variety of virulence factors but also on its great metabolic versatility. *P. aeruginosa* is an aerobic, facultatively anaerobic organism which preferentially obtains its metabolic energy via aerobic respiration and is well adapted to low oxygen concentrations. By controlling the expression of multiple cytochrome oxidases, *P. aeruginosa* appears to exploit the best-suited electron transport chain in response to the available oxygen supply. The genome sequence reveals gene clusters for three cytochrome c oxidases (*ccoNOQP1*, *ccoNOQP2*, and *ccoBA-coIII*) and one quinol oxidase (*cyoABCDE*), all of which belong to the heme-copper superfamily (9, 10, 11). Heme-copper oxidases can be inhibited by cyanide, resulting in a block of electron transport via the cytochrome bd-type cyanide-insensitive oxidase (CIO) (the product of the *cioAB* cluster), which apparently lacks copper in its active site and allows the bacterium to respire oxygen when the other oxidases are inhibited (14).

Here we investigated the adaptation of *P. aeruginosa* to copper limitation under aerobic conditions. The organism’s global transcriptional response reveals that a range of genes involved in iron metabolism and respiration is affected. In a copper-depleted environment, *P. aeruginosa* entirely relies on CIO for aerobic respiration, and CIO expression is markedly induced. Genetic analysis suggests that CIO induction is a consequence of reduced aerobic respiration via the four cyanide-sensitive terminal oxidases.

### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** Strains and plasmids used in this study are listed in Table 1. All media and solutions were prepared with deionized, double-distilled water. Bacteria were routinely grown on nutrient agar and in nutrient yeast broth (NYB) (48) at 37°C. When required, antibiotics were added to these media at the following concentrations: 100 μg/ml for tetracycline, 12.5 μg/ml for tetracycline, 25 μg/ml for kanamycin for *E. coli*, and 300 μg/ml for carbenicillin and 100 μg/ml for tetracycline for *P. aeruginosa*. Growth and β-galactosidase experiments were performed in a minimal medium (OS-glucose) containing 0.5% (wt/vol) glucose, 0.1% (wt/vol) ammonium sulfate, 0.01% (wt/vol) Triton X-100, and salt solutions (38), from which CuSO4 was omitted unless stated otherwise. All glassware was rendered copper free by a 24-h treatment with 0.1 M HCl and rinsed once in double-distilled water before sterilization. A freshly prepared ascorbic acid solution (final concentration, 1 mM) and the copper chelator bathocuproine disulfonic acid (BCS; final concentration, 150 μM; Sigma-Aldrich) were added to OS-glucose when appropriate. Control experiments in which ascorbic acid had been omitted showed that under these conditions the complexation of copper by BCS was incomplete. Growth in OS-glucose medium was obtained in 100-ml Erlenmeyer flasks filled with 20 ml of medium, under conditions of good aeration (shaking at 180 rpm) at 37°C.

### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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**Strains**

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<td>E. coli DH5α</td>
<td>recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1 Δ(lacZYA-argF)U169 [φ80d aziΔM15] F− ρalr</td>
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<tr>
<td>HB101</td>
<td>proA2 hsdS20(rB mB ) lacZ15lacY1 galK2 rpsL20 supE44 xyl-5 mtl-1 F−</td>
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**Plasmids**

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<tr>
<td>pME3087</td>
<td>Suicide vector for allelic replacement; Tc− ColE1 replicon</td>
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<td>pME6015</td>
<td>Cloning vector for translational lacZ fusions; Te−</td>
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<td>pME6016</td>
<td>Cloning vector for transcriptional lacZ fusions; Te−</td>
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<td>pME6031</td>
<td>Expression vector carrying pac lacF−; Te−</td>
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<td>pME6031 derivative carrying the cioAB genes; Tc−</td>
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<td>Suicide construct used for deletion of the two adjacent ccoNOQP operons; Tc−</td>
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Determination of cellular copper concentrations. Total copper in whole cells was measured by inductively coupled plasma mass spectrometry (Hewlett-Packard 4500; Agilent Technologies, Palo Alto, CA). P. aeruginosa PAO1 was grown in OS-glucose medium with vigorous shaking at 37°C for 14 h. This culture was diluted 1:200 in 200 ml of the same medium supplemented with acetic acid plus BCS, acetic acid alone, or 1.5 μM CuSO4. The cultures were harvested in exponential growth phase (optical density at 600 nm [OD600] = 1) by centrifugation at 15,000 × g for 15 min, washed twice with 5 ml 0.5% NaCl, digested with 500 μl of low-metal-content concentrated HNO3 (Baker instra grade) at 95°C for 1 h, and then diluted with 4.5 ml of double-distilled water before the measurements were performed. The instrument was calibrated using a standard CuSO4 solution. In parallel, viable counts (CFU/ml) were measured for the bacterial cultures. This allowed us to estimate the number of copper atoms/viable P. aeruginosa cell.

Construction of plasmids and gene replacement mutants. DNA cloning and plasmid preparations were performed according to standard methods (45). Large-scale preparations of plasmid DNA were performed using JETstar 2.0 (Genomed). Restriction and DNA-modifying enzymes were used following the instructions of the manufacturers. All oligonucleotide primers used below are listed in Table S1 in the supplemental material. A transcriptional ciao-lacZ fusion, in which the +1 nucleotide of lacZ was fused to the major +1 start site of the ciao promoter (14), was constructed by cloning a 410-bp fragment containing the ciaoA promoter region into the EcoRI-BamHI sites of plasmid pME6016. This fragment was generated by PCR using the P. aeruginosa PAO1 genome as the template and primers ciaoA-Pa1 and ciaoRV2. A translational ciaoA-lacZ fusion was constructed by inserting a 621-bp BglII-EcoRI fragment carrying the proximal part of ciao into the BamHI-EcoRI sites of plasmid pME6015. This fragment was generated with the amplification of the proximal part of the ciaoA gene by use of primers ciaoA-Pa1 and ciaoA-Pa2. A translational pvdS-lacZ fusion was constructed similarly by fusing a 0.65-kb EcoRI-BamHI fragment carrying the proximal part of pvdS with its own promoter (amplified from the PAO1 genome with primers PvdPSEF and PvdPPSR) to ‘lacZ’ in pME6013.

For the inactivation of the ciaoA operon in the P. aeruginosa PAO1 chromosome, a 624-bp fragment overlapping ciao and a 617-bp fragment overlapping ciaoB were amplified by PCR using primers ciaoA-Pa1/ciaoA-Pa2 and ciaoB-Pa1/ciaoB-Pa2, respectively. These products were digested with EcoRI-BglII and BglII-HindIII, respectively, and cloned into the corresponding sites of the suicide vector pME3087, giving plasmid pME7541. Plasmid pME7541 was then used to create copper limitation in a P. aeruginosa complemented with the pvdS-lacZ fusion was constructed similarly by fusing a 0.65-kb EcoRI-BamHI fragment carrying the proximal part of pvdS with its own promoter (amplified from the PAO1 genome with primers PvdPSEF and PvdPPSR) to ‘lacZ’ in pME6013.

For the deletion of ciaoA (PA0105), ciaoA (PA0106), PA0107, and ciaoB (PA0108) (17, 50), a 1,316-bp fragment overlapping ciaoA and a 1,088-bp fragment overlapping ciaoB were amplified by PCR using primers ciaoFP1/ciaoRPV and ciaoBFW/ciaoBFW, respectively. These products were digested with BamHI-EcoRI and EcoRI-HindIII, respectively, and cloned into pME3087, giving plasmid pME9302. Plasmid pME9302 was then used as described above to produce strain PAO6937, giving plasmid pME9307. A double mutant (PA6957) deleted for the cos and cyoABCDE clusters was obtained as follows. A 1.060-bp fragment overlapping cyoA and a 1.088-bp fragment overlapping cyoE were amplified by PCR using primers cyoAFW/cyoxRPV and cyoxFW/cyoxFW, respectively. These products were digested with BamHI-EcoRI and EcoRI-HindIII, respectively, and cloned into pME3087, giving plasmid pME9307, which served to construct strain PAO6957. A quadruple mutant carrying a deletion of all four operons encoding heme-copper terminal oxidases was derived from PAO6957 as follows. A 1,287-bp fragment overlapping ccoN (PA2815) and a 1,280-bp fragment overlapping ccoP (PA2815) were amplified by PCR using primers ccoFP1/ccoxRPV and ccoNFW/ccoxFW, respectively. These products were digested with BamHI-EcoRI and EcoRI-HindIII, respectively, and cloned into pME3087, resulting in plasmid pME9308. Plasmid pME9308 was then used to introduce P. aeruginosa PAO1 as described above; after the excision of the integrated plasmid strain, PAO6959 (ΔcoxRΔsr) was obtained.

A copRS (PA2809-PA2810) mutant of PAO1 was constructed by amplifying a 993-bp fragment carrying copR and a 615-bp fragment carrying copS with primers copUPW/copRPV and copFW/copDWR, respectively. These products were cut with EcoRI-BamHI and BamHI-HindIII, respectively, and cloned into pME3087, resulting in plasmid pME7576, which was used to generate strain PAO6573 (ΔcopRS) as described above. For all mutants described here, the deletions were confirmed by PCR and PCR fragments were checked by sequencing.

β-Galactosidase assays and pyoverdine determination. β-Galactosidase assays (35) were performed with P. aeruginosa cultures grown in triplicate in OS-glucose medium. Data are mean values for three independent samples ± standard deviations. PyoverdinePAG was quantified by measuring the absorbance at 405 nm of culture supernatants diluted 9:1 in 100 mM Tris-HCl (pH 8.0) per cell population density (in OD600 units) as previously described (53).

RNA isolation, generation of cDNA probes, and transcriptome analysis. P. aeruginosa PAO1 was inoculated at an OD600 of 0.01 into 20 ml of OS-glucose medium supplemented with 1 μM of acetic acid, with or without 150 μM BCS. The cultures were grown at 37°C with vigorous shaking, until they reached an OD600 of approximately 1, and then cells were harvested and RNAProtect Bacteria (Qiagen) were added. Total RNA was isolated by the hot phenol method as described elsewhere (31), followed by DNase I treatment (Roche). The integrity of total RNA was confirmed by agarose gel electrophoresis and an RNA 6000 Nano LabChip in an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). Next, 10 μg of total RNA was used with random primers and Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA) to perform cDNA synthesis. cDNA fragmentation, labeling, hybridization, staining, and washing steps were performed according to the manufacturer’s protocol for the Affymetrix P. aeruginosa GeneChip arrays (Affymetrix, Inc., Santa Clara, CA). Finally, the arrays were scanned with the Affymetrix GeneChip scanner 3000.

Processing of the P. aeruginosa GeneChip (Affymetrix) was performed at the University of Lausanne Center for Integrative Genomics. For each condition, cultures were grown in triplicate, and RNAs from these cultures were pooled before proceeding to cDNA synthesis. In addition, biological replications for each condition were performed on a separate day and run on a different microarray chip. We refer to the “most strongly induced or repressed genes” as those genes meeting the following criteria: (i) the P value obtained for each transcript analysis is less than 0.05 and (ii) the absolute change in the transcript level is equal to or greater than twofold.

RESULTS

Copper depletion affects the expression of genes involved in iron metabolism of P. aeruginosa. To assess the effect of a copper-depleted environment on the transcriptional expression of P. aeruginosa genes, we developed a defined medium (OS-glucose) containing BCS, a specific, high-affinity Cu(I) chelator (Kd 10−20 M [6, 43]). BCS has previously been used to create copper limitation in a Synecochystis sp. and in yeast (17, 50). Unless stated otherwise, OS-glucose medium was not amended with copper salts, contained an excess of iron (10 μM FeSO4), and was supplemented with 1 mM ascorbic acid (freshly prepared) to reduce any trace of Cu(II) present to Cu(I) (18). Various BCS concentrations were tested to determine the highest level of BCS that could be added to liquid OS-glucose without affecting the growth rate of the wild-type P. aeruginosa PAO1. When strain PAO1 was grown in this medium containing 150 μM BCS, its growth rate was indistinguishable from that observed for the unamended medium (data not shown). Ascorbate was omitted in those experiments where CuSO4 was added because ascorbate would have exacerbated copper toxicity. Growth was achieved in shake flasks under conditions of good aeration; therefore, oxygen was not a growth-limiting factor.

Inductively coupled plasma mass spectrometry analysis re-

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### TABLE 2. List of 142 *P. aeruginosa* genes most strongly induced or repressed in response to copper starvation

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<tr>
<th>Gene (name)</th>
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<tr>
<td>PA0460</td>
<td>3.0</td>
<td>Hypothetical protein</td>
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<td>PA0918</td>
<td>2.8</td>
<td>Cytochrome b₅₆₅</td>
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<td>PA1502 (<em>acrA</em>)</td>
<td>2.1</td>
<td>Aconitate hydratase 1</td>
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revealed that wild-type *P. aeruginosa* PAO1 cells, when grown in the presence of BCS for six generations, displayed a significant decrease in the cellular copper concentration (1,000 ± 400 Cu atoms per viable cell) compared with that found for the unamended culture (38,000 ± 7,000 Cu atoms per viable cell) and with that found in a culture supplemented with 1.5 μM CuSO₄ (360,000 ± 70,000 Cu atoms per viable cell). The value measured for cells grown in BCS medium was close to the detection limit and represents a maximal estimate. These results, obtained from three independent experiments with strain PAO1, show that BCS can be used to reduce the cellular copper content to very low levels and also support previous observations that *P. aeruginosa* has homeostatic regulatory mechanisms preventing an overload of cellular copper (49). In a control experiment, we verified that the complexation of copper by BCS and the addition of excess copper did not cause significant changes in cellular iron, zinc, nickel, manganese, molybdenum, and cobalt (data not shown).

To search for genes differentially regulated during copper starvation versus copper sufficiency, we performed a transcriptional analysis of *P. aeruginosa* cells grown to exponential phase (OD₆₀₀ = 1.0) in the presence or absence of BCS. Among the 5,901 genes represented on the Affymetrix chip, 132 genes exhibited a ≥2-fold increase in transcript levels, whereas only 10 genes showed a ≥2-fold increase in mRNA levels (Table 2). Copper starvation resulted in a strong decrease of the expression of many genes and of operons that play a role in siderophore-mediated iron acquisition (13, 42, 52), including genes for pyoverdine biosynthesis (*pvdS* for the master regulator and sigma factor PvdS, *pvdA*, *pvdQ*, *pvdP*, *pvdNO*, *pvdF*, *pvdE*, *pvdID*, *pvdH*, *pvdLG*), pyochelin biosynthesis (*pchR* for the pathway-specific regulator PchR, *pchDCBA*), the corresponding siderophore receptors (*fpvA* and *fpvB*; *fpvA*), and a TonB protein (PA5531) (Table 2). Copper starvation also diminished the expressions of genes involved in heme uptake and metabolism (*hasA, hasR, phuT, phuR, hxxC*, and *hemO*) and heterologous siderophore uptake (*pfeR, pfeS*, and *pfeA; pirA; chtA; foxA, foxR, and foxI*) and of the manganese-cofactored superoxide dismutase (*sodA*) and furamur (fumCI) genes (Table 2). In general, the expression of these genes is known to be repressed by the ferric uptake regulator Fur in the presence of iron (13, 22, 42, 52). Surprisingly, few genes were expressed at elevated levels during copper starvation. Among such genes, we noted *bfrB*, encoding the iron storage protein bacterioferritin (Table 2). Altogether, there was a strong overlap between *P. aeruginosa* genes responding to copper starvation (Table 2) and those regulated by iron depletion (12, 37, 40), although copper and iron limitations have opposite effects on the expression of these genes.

### Table 2—Continued

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*ECF, extracytoplasmic function.*
ble 3). BCS addition also caused a significant reduction in the expression of the pchR'-lacZ fusion (Table 3). Thus, copper limitation also had a negative impact on siderophore expression in *P. aeruginosa* under conditions of poor iron availability. Previous work indicates that both pyoverdine and pyochelin of *P. aeruginosa* are able to chelate not only Fe(III) but also Cu(II) (53, 57), although it is questionable whether these siderophores can actually promote copper uptake. Our data argue against a scenario in which copper-depleted cells would induce siderophore production to enhance copper uptake.

**Copper availability influences the expression of aerobic respiratory pathways in *P. aeruginosa***. The transcriptome data (Table 2) reveal that two genes of the quinol oxidase operon *cyoABCDE*, *cyoB* (PA1318) and *cyoC* (PA1319), were downregulated, whereas two genes encoding uncharacterized cytochromes (PA0918 and PA5300) were upregulated during conditions of copper starvation. The *cyoABCDE* cluster encodes a putative boT-type quinol oxidase (55), which appears to function as a low-affinity terminal oxidase under high-oxygen conditions (2). Furthermore, for copper-starved cells we observed a two- to threefold downregulation in the expression of 16 genes involved in the type III secretion apparatus (*pcvV*, *pcrH*, *popB*, *popD*, *esC*, *esE*, *esA*, *esD*, and *pscBCEFGHJ*) (Table 2), which can probably be attributed to the reduced expression of the PvdS sigma factor and, consequently, of the major ExsA regulator (12). While we did not follow up the effect on type III secretion genes, we decided to investigate the impact of copper starvation on respiratory functions in more detail. Assuming that copper is an essential cofactor of the *cyo*, *cox*, *ccoI*, and *cco2* terminal oxidases, we reasoned that during copper starvation these oxidases would not function properly and that *P. aeruginosa* would mainly rely on the CIO—which does not contain Cu—to respire oxygen. Given that the transcriptional expression of *cco* oxidase was downregulated (Table 2), we expected to see a compensating upregulation of the expression of the alternative quinol oxidase CIO. Although such an effect was not evident from the microarray data (taken at an early growth phase), we found that a transcriptional *cioA-lacZ* fusion carried by pME9306 was upregulated in strain PAO1 grown in OS-glucose medium during conditions of copper limitation, especially during early growth phases (Fig. 1A). As the β-galactosidase expression of this fusion was extremely high, this experiment may not reflect the full extent of upregulation during later growth phases. A translational *cioA*-lacZ fusion (on pME7556), which specified lower β-galactosidase activities, showed upregulated expression throughout growth under copper limitation (Fig. 1B). In unsupplemented OS-glucose medium, the expression of both fusion constructs increased in a growth-phase-dependent manner, as previously observed by Cooper et al. (11). An excess (1.5 μM) of CuSO4 strongly repressed the expression of both fusions (Fig. 1A and B). To show that this regulation was specific, we measured the expres-

**FIG. 1.** Activities of β-galactosidase reporter plasmids containing either a *cioA-lacZ* transcriptional fusion (A) or a *cioA*-lacZ translational fusion (B) in wild-type strain PAO1. Cultures were grown aerobically in OS-glucose medium containing 1 mM ascorbate (diamonds), the ascorbate medium amended with 150 μM BCS (squares), or medium with 1.5 μM CuSO4 but without ascorbate (triangles). Each value is the average of three different cultures ± standard deviation.
sion of a constitutive housekeeping gene, proC (46), under the same conditions. We found that the expression level of a trans-/H11006-mained constant at 800 standard deviation. Each value is the average of three different cultures.

To demonstrate the pivotal function of CIO during copper limitation, we constructed a shift experiment. The wild-type PAO1, the rioAB deletion mutant of P. aeruginosa PAO1 (PAO6437) and tested its ability to grow in OS-glucose medium with or without the addition of BCS. Without BCS, the mutant PAO6437 and the wild-type PAO1 showed similar growth rates (Fig. 2A). The addition of BCS strongly inhibited the growth of strain PAO6437 but had little effect on wild-type PAO1 and on the complemented mutant PAO6437/pME9305 (Fig. 2A). In the complementing plasmid, pME9305, the rioAB operon is under the control of its own promoter. The addition of 2 μM CuSO4 to the medium containing BCS fully restored the growth of the rioAB mutant PAO6437. By contrast, the addition of 10 μM ZnSO4 did not restore growth in the presence of BCS and had no effect in the absence of BCS (Fig. 2B). Taken together, these results show that CIO is essential for aerobic growth of P. aeruginosa during copper limitation.

We confirmed the copper requirement of the rioAB mutant on solid OS-glucose medium by using another specific Cu(I) chelator, tetrathiomolybdate (TTM) (5, 30). In the presence of 1 mM TTM, the wild-type PAO1 grew, whereas the rioAB mutant was completely inhibited. The addition of CuSO4 overcame this inhibition partially at 30 μM and entirely at 300 μM (Fig. 3).

Copper-mediated regulation of CIO does not rely on the two-component systems RoxSR and CopRS. Previous studies revealed that CIO expression is positively controlled by the two-component system RoxSR (9). Moreover, another two-component system, CopRS, is known to be involved in resistance to copper stress in P. aeruginosa (21, 49). To test a potential role of these two-component systems in the regulation of CIO by copper, we constructed rioSR and copRS deletion mutants of P. aeruginosa termed PAO6594 and PAO6573, respectively. The rioSR mutant, similar to the rioAB mutant, showed reduced growth in OS-glucose in the presence of BCS (data not shown). Therefore, to study CIO expression in the rioSR mutant during copper limitation, we performed a shift experiment. The wild-type PAO1, the rioSR mutant PAO6594, and the copRS mutant PAO6573 were transformed with pME7554 (carrying the translational rioA::lacZ fusion) and grown in OS-glucose to an OD600 of approximately 0.8. Then, cultures were split and challenged with BCS or with 1.5 μM CuSO4 (Fig. 4) or left untreated. For the rioSR mutant, we observed reduced CIO expression, confirming results previously reported by Comolli and Donohue (9). In the copRS strain, CIO expression was comparable to that observed for the wild-type strain PAO1. In all three strains, the addition of copper caused a 2- to 3-fold decrease in CIO expression compared to what was seen for the control, whereas the addition of BCS caused a minor (1.5-fold) upregulation of CIO expression. These data indicate that the mechanism controlling
expression in relation to copper availability is independent of the two-component systems RoxSR and CopRS. The fact that the BCS effect was less pronounced in the shift experiment (Fig. 4) than in the batch experiment (Fig. 2B) suggests that BCS depleted the cellular copper reserves more slowly in the dense populations prior to the shift compared to what was seen for the initial very low population densities used in the batch experiment.

**Copper-mediated regulation of CIO appears to be indirect.** It has been proposed that the electron flow toward the four terminal oxidases belonging to the heme-copper family indirectly regulates the expression of CIO (9). One signal involved in this regulation may be hydrogen cyanide, which acts as an inhibitor of the heme-copper oxidases (11). We reasoned that a lack of copper might act like cyanide. In the absence of copper, the heme-copper oxidases would be unable to transfer electrons to oxygen and the resulting reduced electron flow might activate CIO expression. To test this hypothesis, we constructed a quadruple mutant, PAO6650 (ccoNOQP1 ccoNOQP2 coxBA-coIII cyoABCDE), lacking all four heme-copper oxidases. The growth of PAO6650 (doubling time, 80 min) was slower than that of the wild-type PAO1 (doubling time, 30 min) in NYB, and after 9 h of incubation, the growth yield of PAO1 was 1.5-fold higher than that of PAO6650. Nevertheless, it is remarkable that the CIO enzyme alone can support aerobic growth of *P. aeruginosa*.

In the quadruple mutant, ciaoA-lacZ expression increased in a growth-phase-dependent manner (Fig. 5), much like what was seen for the parental strain PAO1 (Fig. 2B). Interestingly and unlike the wild type, the quadruple mutant did not show any negative effect on CIO expression upon the addition of copper (Fig. 5). The addition of BCS caused an initial and transient increase of CIO expression relative to what was seen for the untreated culture (Fig. 5). However, this unexplained effect disappeared at later growth phases. At high population densities (OD<sub>600</sub> = 3), the availability of copper had no significant effect on ciaoA-lacZ expression in the quadruple mutant (Fig. 5), whereas in the wild type there was a 10-fold expression difference between copper-replete and copper-limited conditions (Fig. 2B). These data show that copper-mediated regulation of CIO depends to a large extent on the function of the heme-copper oxidases.

We wondered whether siderophore regulation by copper availability might be altered in the quadruple oxidase-negative mutant PAO6650. However, the expression of the pwsS-lacZ and pchR-lacZ fusions was still downregulated by copper limitation, as in the wild-type PAO1 (Table 3). We conclude that respiratory functions are not involved in the link between iron metabolism and copper availability.
DISCUSSION

We have shown here that the P. aeruginosa wild-type PAO1 can perform aerobic respiration when very little, if any, copper is bioavailable. Only when the cioAB genes were inactivated by mutation did the organism require copper for aerobic growth, and this requirement could not be satisfied by iron or zinc. These results are consistent with genomic data which predict that aerobic respiration of P. aeruginosa depends on four heme-copper terminal oxidases (encoded by the ccoNOQP1, ccoNOQP2, coxA-coIII, and cyoABCDE clusters) and one copper-free, cyanide-resistant oxidase (encoded by the cioAB genes). Thus, when copper is limiting, P. aeruginosa essentially relies on CIO for aerobic growth. Interestingly, as shown by the quadruple cco1 cco2 cox cyo mutant, the CIO pathway alone seems to allow fairly good growth of P. aeruginosa, implying that protons are translocated and that ATP is generated effectively in this pathway. Alternative cyanide-resistant ubiquinol oxidases also exist in plants and some fungi. Whereas in plants these enzymes do not conserve energy, those in fungi appear to be able to do so (4, 26).

BCS probably does not penetrate cells and therefore depletes them of copper progressively during growth. Therefore, we conducted a serial transfer experiment (not shown) in which we grew P. aeruginosa PAO1 in OS-glucose medium with BCS. As growth continued normally for at least 20 generations, we believe that copper is not essential for aerobic growth. We confirmed this finding by using the potent permeable copper chelator TTM. On defined medium containing 1 mM TTM, the wild-type PAO1 was able to grow, whereas the cioAB mutant was not (Fig. 3). We did not assess the effect of copper limitation during anaerobic respiration with nitrate or nitrite, which P. aeruginosa can use as alternative electron acceptors (60). In P. aeruginosa, as in P. stutzeri, the last enzyme of denitrification, N₂O reductase, is a copper protein (33, 60), and it is conceivable that a truncated form of denitrification ending with N₂O might operate under conditions of copper limitation. In the absence of respiration, P. aeruginosa is also capable of marginal anaerobic growth on arginine by fermenting arginine via the arginine deiminase pathway (51). Further experiments will be needed to see how copper availability affects these processes.

In P. aeruginosa, stress imposed by high copper concentrations induces the expression of copper resistance genes regulated by the two-component system CopSR and several efflux genes as well as the pyoverdine biosynthetic genes (49). Our transcriptomic data (Table 2) show that, conversely, copper deficiency results in the downregulation of the pyoverdine biosynthetic genes and pyoverdine production. It is possible that pyoverdine, by chelating Cu(II) in culture media, might alleviate copper toxicity to some extent, although there is no experimental evidence for this. We also found that pyochelin biosynthetic genes were downregulated during copper deprivation. These data are difficult to reconcile with a previous study (49) showing that downregulation of the same genes occurs during copper stress. It is striking that many P. aeruginosa genes whose expression is low under copper limitation (Table 2) are involved in iron metabolism, suggesting mechanistic links between iron and copper metabolism. Such links have been noted before for E. coli (28), yeast (50), and mammals (56). In P. aeruginosa, we verified that the genes for two key regulators of iron uptake, the sigma factor PvdS and the pyochelin regulator PchR, were very poorly expressed during copper limitation, both in high- and low-iron media (Table 3). We do not know at this stage what causes this effect. Although the E. coli Fur protein binds Cu²⁺ ions and thereby is converted to a repressor in vitro (15), it is unlikely that such a mechanism operates in P. aeruginosa in vivo. If it did, we would expect to find derepression of the Fur-repressible pvdS and pchR genes under copper-limiting conditions. However, the opposite effect was observed.

We found amazingly few genes that were upregulated by copper deprivation in P. aeruginosa (Table 2). These results argue against the existence of P. aeruginosa of an inducible, chalkophore-dependent copper uptake system of the kind that delivers copper to copper-starved cells of some methylotrophs (29). As long as P. aeruginosa can rely on CIO function for respiration, this bacterium may not need an expensive copper-scavenging system. When respiration is curtailed because of a lack of copper, the cell has a reduced requirement for iron. This is reflected, on the one hand, by the enhanced expression of the bacterioferritin gene bfrB and, on the other hand, by the downregulation of multiple iron uptake systems. A potential link between copper and iron regulation might be provided by the PA2384 gene, whose expression was decreased 17-fold by copper limitation (Table 2). This gene is presumed to code for a DNA-binding protein which positively regulates PvdS and PchR expression (59).

Copper availability affected the expression of several respiratory enzymes. In particular, we found that both transcriptional and translational cioA-lacZ fusions were markedly upregulated during copper deprivation and downregulated by excess copper (Fig. 1). It is not clear why this effect was not revealed by our transcriptomic data. A possible explanation could be that in the control culture grown without BCS the cioAB transcript levels were already very high (reflected by the high β-galactosidase activities of the transcriptional lacZ fusion), such that a further enhancement of these mRNA levels upon BCS addition might be difficult to pick up by hybridization. In fact, the plasmid-borne transcriptional lacZ fusion also seemed to arrive at a ceiling during exponential growth with BCS and did not increase further at later growth phases, whereas the less strongly expressed translational lacZ fusion did. Neither the CopSR nor the RoxSR two-component system appeared to be important for copper-dependent regulation of the cioAB cluster. By contrast, in the quadruple cco1 cco2 cox cyo mutant, the extent of this regulation was strongly diminished, especially at high cell population densities, suggesting that some function of the heme-copper oxidases accounts for the copper-dependent regulation of the cioAB genes, at least in part. Whatever signal might be emitted by the heme-copper oxidases, this signal does not appear to be sensed by the CopSR and RoxSR two-component systems. From a physiological perspective, it makes sense that the wild type should respond to copper deprivation by cioAB overexpression, as this optimizes the potential for aerobic respiration.

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