Mutation or Overexpression of a Terminal Oxidase Leads to a Cell Division Defect and Multiple Antibiotic Sensitivity in *Pseudomonas aeruginosa*  

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Mutant of the cyanide-insensitive terminal oxidase of *Pseudomonas aeruginosa* leads to pleiotropic effects. A *cio* mutant and strains, including the wild-type, carrying the *cioAB* genes on a multicopy plasmid were temperature-sensitive and had a cell division defect, leading to the formation of non-septate, multinucleated filaments. Such strains of this intrinsically antibiotic-resistant bacterium were more sensitive to a range of antibiotics including chloramphenicol, β-lactams, quinolones, aminoglycosides, and macrolides. The effect of *cio* mutation on Δp-dependent accumulation of chlorophyll suggested that antibiotic sensitivity resulted from loss of or damage to a multidrug efflux pump. The ability of reducing agents and catalase to suppress the temperature-sensitive phenotype and of catalase to partially suppress antibiotic sensitivity suggested that increased levels of reactive oxygen species might be the cause of the observed phenotypes. Consistent with this was the increased sensitivity of strains to H₂O₂ and their increased protein carbonyl content, an indicator of oxidative protein modification. The temperature-dependent synthesis of a specific catalase was absent in the *cio* mutant and in strains carrying multiple plasmid-borne copies of *cioAB*. We propose that reduced catalase levels result in oxidative modification and consequent loss of function of proteins involved in a range of cellular functions. How mutation or overexpression of the cyanide-insensitive terminal oxidase leads to a loss of catalase activity is unknown at present.

Respiratory adaptability is likely to be an important component of the growth and survival of *Pseudomonas aeruginosa* in the diverse ecological niche in which it is found. This Gram-negative, opportunistic pathogen is able to respire and grow under a variety of aerobic and anaerobic conditions (1). Although it preferentially obtains energy via aerobic respiration, it is well adapted to conditions of limited oxygen supply (2). It is capable of anaerobic growth with nitrate as a terminal electron acceptor, and in its absence it is able to ferment arginine, suggesting that it is well adapted to conditions of limited oxygen supply (2). It is well adapted to conditions of limited oxygen supply (2). It is well adapted to conditions of limited oxygen supply (2). It is well adapted to conditions of limited oxygen supply (2). It is well adapted to conditions of limited oxygen supply (2). It is well adapted to conditions of limited oxygen supply (2). It is well adapted to conditions of limited oxygen supply (2). It is well adapted to conditions of limited oxygen supply (2). It is well adapted to conditions of limited oxygen supply (2).

Received for publication, October 9, 2002

Published, JBC Papers in Press, November 14, 2002, DOI 10.1074/jbc.M210355200
at concentrations that inhibit terminal oxidases of the heme-copper superfamily (9). Therefore, it has been proposed that the production of the Cio is an important adaptation, allowing aerobic respiration under cyanogenic conditions (5). Cyanide has been detected in tissue samples infected with *P. aeruginosa* (10), and it has been shown recently (11) that cyanide poisoning is responsible for nematode killing in one of the *C. elegans* virulence models. It is established that defects in the cytochrome bd of *E. coli* can lead to a range of phenotypes, including temperature sensitivity for growth and difficulty in exiting stationary phase (12-16). Therefore, we were interested in whether mutation of the Cio had pleiotropic effects on *P. aeruginosa*. We show that mutation or introduction of the ciao genes on a multicopy plasmid leads to a range of phenotypes in *P. aeruginosa* including a cell division defect and multiple antibiotic sensitivity via loss or damage to a Δp-dependent efflux pump.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids—** *P. aeruginosa* PAO6049 met–9011 amiE200 strA was used as the wild-type strain in these studies, together with the ciao mutant PAO7701 ciao–miniD-171, which has been described previously (5, 6). The plasmids used to complement these strains are described in Table I.

**Growth Conditions—** Bacteria were grown in LB medium (17) with agar added to 1.5% (w/v) for solid medium. As required, antibiotics were added to the following concentrations: streptomycin (1 mg/ml), tetracycline (300 μg/ml), and carbenicillin (500 μg/ml). Starter cultures were prepared by inoculating a single colony from a LB plate into 5 ml of LB broth in a 25-ml sterile universal bottle and incubating overnight at 30 °C in an orbital shaker at 200 rpm. Overnight cultures were diluted 1:100 (into 50 ml of LB in a 250-ml conical flask, to give an A600 of 0.02–0.04) for growth at 30 and 37 °C or diluted 1:25 to (to give an A600 of 0.1) for growth at 42 °C, and cultures were incubated with shaking at 200 rpm. Membranes were prepared as described previously (5). Protein content was determined by the method of Markwell et al. (18). Phosphate-buffered saline contained (in g liter−1) NaH2PO4·H2O (2.6), Na2HPO4 (11.5), and NaCl (8.5).

**Investigation of the Temperature-sensitive (Ts) Growth Phenotype—** Strains were grown aerobically on LB agar plates supplemented with appropriate antibiotics and incubated at 30, 37, or 42 °C for 2 h. For broth cultures bacteria were grown overnight in LB broth at 30 °C, diluted 1:100 into LB to give an A600 of 0.02–0.04, and incubated at 30, 37, or 42 °C. The effects of various reducing agents and non-reducing agents on the Ts phenotype was determined as described by Goldman et al. (19). The following compounds were used at the concentrations described previously (19): reduced glutathione, methionine, cysteine, 1-cysteine, 0-cysteine, glutamine, oxidized glutathione, mercaptoethanesulfonic acid, a combination of dithiothreitol and oxidized glutathione, bovine catalase, and bovine superoxide dismutase. They were added to 1-cm filter disks at the center of seeded plates, and the plates were incubated at 42 °C for 24 h.

**Hydrogen Peroxide Sensitivity Assay—** Cultures were grown in LB medium until mid-exponential phase (A600 = 0.6) at 37 °C before challenging with a final concentration of 10 mM hydrogen peroxide. Samples were collected at different time points, and after dilution in LB they were plated onto LB agar and incubated at 30 °C to obtain viable cell counts.

**Microscopy—** A 1-ml sample of a stationary phase culture was fixed and stained with 4',6-diamidino-2-phenylindole as described by Hiraga et al. (20) and observed using a Zeiss Axiovision 3.0 microscope, and images were captured using an AxioCam digital camera and processed with Zeiss Axiovision software.

**Determinations of Minimal Inhibitory Concentrations (MIC)—** The cultures were grown to stationary phase at 30 or 37 °C. They were harvested and diluted to 5 × 106 cell ml−1 in medium containing the test antibiotic (1 ml of medium in a 10-ml tube). Tubes were incubated overnight at 30 or 37 °C with shaking. The MIC corresponded to the minimum concentration of drug that could prevent the growth of cells, as determined by visible inspection. As the ciao mutant and strain carrying pLC8 or pLC5 grew at 42 °C have difficulty exiting stationary phase a modified procedure was used to determine MICs at these temperatures. Cultures were initially grown to stationary phase at 37 °C and then diluted into LB to a cell concentration of 2.5 × 105 cell ml−1 containing appropriate concentrations of the test antibiotic and then incubated at 42 °C. The MICs were read after 24 h of incubation.

**Assay of Chloramphenicol Accumulation in Intact Cells—** Cells from stationary phase cultures at 30 and 37 °C were harvested and diluted in fresh LB. The cultures were grown to mid-exponential phase, when the culture density had reached 0.25–0.4 mg (dry weight) ml−1. To assay drug accumulation at 42 °C, a culture was first grown to stationary phase at 37 °C, subcultured, and grown to mid-exponential phase at 42 °C. Chloramphenicol uptake experiments were performed on these cells using [3H]chloramphenicol as described by Li et al. (21), except that a different mixture of silicone oils (a 4:6 (v/v) mixture of Dow Corning silicone oils 510 and 550) was used to separate cells from the supernatant fraction prior to determination of radioactivity in the cell fraction by liquid scintillation counting.

**Hydrogen Peroxide Production—** Cells from 2.5 ml of a culture grown in LB to early stationary phase were harvested and resuspended in 25 ml of phosphate-buffered saline. After 20 min, samples were centrifuged at 4500 × g for 5 min to pellet the cells, and the hydrogen peroxide concentration of the supernatant was determined using the scopoletin assay as described (22), except that the reaction mix contained 0.1 μM scopoletin instead of 0.2 μM scopoletin.

**Determination of Catalase Activity—** Catalase activity was determined in total cell lysates (4 μg of protein per sample) using a Clark-type oxygen electrode (23). Catalase activity in polyacrylamide gels was localized as described previously (24).

**Oxidative Protein Modification Assays—** The carbonyl groups in the protein side chains were derivatized, using the OxyBlot kit (Intergen), to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine. Total lysates were prepared from cultures grown in LB to early stationary phase and slot-blotted using a 48-well apparatus (Schleicher & Schuell) onto nitrocellulose membranes. Oxidatively modified proteins were detected with anti-2,4-dinitrophenylhydrazine antibodies using the ECL detection reagents (Amersham Biosciences).

**RESULTS**

**Mutation or Overexpression of the Cyanide-insensitive Oxidase Leads to Temperature-sensitive Growth—** The wild-type (PAO6049), a ciao mutant (PAO7701), and a complemented mutant (PAO7701/pLC8) all grew similarly at 30 °C. We have shown previously that pLC8 complements PAO7701 for its respiratory defect (5). Both the mutant and complemented mutant grew more slowly than the wild-type at 37 °C, but on solid medium they form similarly sized colonies (−1.5−2.0-mm diameter). The wild-type grew more rapidly at 42 °C, but both the mutant and the complemented mutant grew very poorly at 42 °C, forming only pinprick-sized colonies on solid medium (<0.1-mm diameter compared with 1.5−2.0-mm diameter for the wild-type). In broth cultures both the ciao mutant (PAO7701) and the complemented mutant (PAO7701/pLC8) failed to grow promptly at 42 °C when our usual inoculum (A600 = 0.02) was used (Fig. 1). However, if a larger inoculum (A600 = 0.1) was used then the mutant grew in liquid culture, albeit after a significant lag (data not shown). However, the Ts
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Table I

| Plasmids | Relevant properties | Exponential phase CIO activity when indicated plasmid is present in strain | Stationary phase CIO activity when indicated plasmid is present in strain | References |
|----------|---------------------|------------------------------------------------------------------|------------------------------------------------------------------|------------|
| No plasmid |                     | PAO6049 26 1 134 ± 11 22 ± 4 | PAO6049 26 1 134 ± 11 22 ± 4 |            |
| pLC1     | Based on the in vivo cloning plasmid pADD386, TetR | 40 ± 2 2 ± 1 134 ± 11 22 ± 4 | 40 ± 2 2 ± 1 134 ± 11 22 ± 4 |            |
| pLC2     | A pUCP18 derivative containing a 5.2-kb HindIII fragment including cioA, CbR | 34 ± 2 40 ± 9 154 ± 18 130 ± 22 | 34 ± 2 40 ± 9 154 ± 18 130 ± 22 | 37         |
| pLC8     | A pUCP18 derivative containing a 2.7-kb fragment carrying only cioA and cioB, CbR | 58 ± 2 55 ± 9 115 ± 22 104 ± 10 | 58 ± 2 55 ± 9 115 ± 22 104 ± 10 | 5 and 6    |

growth phenotype was not because of a polar effect of the mini-D171 insertion used to construct the cio mutant strain (6), as introducing pLC8 or pLC2 conferred the Ts growth phenotype upon the wild-type (Fig. 1) (data not shown). Introduction of the cioAB genes into the wild-type strain led to intermediate growth phenotype at 42 °C (Fig. 1). The plasmid pLC8 carries a 2.7-kb fragment in which the only open reading frames are the cioA and cioB genes. We have shown previously that complementation of the mutant with the cioAB genes on a multicopy plasmid or introduction of the plasmid into the wild-type led to an overexpression of CIO activity compared with the wild-type strain (5, 6), whereas the plasmid pLC1 had a much less marked effect. Therefore, it seemed plausible that overexpression of CIO activity may lead to the defective growth phenotype at 42 °C. Most of these earlier experiments were done on late exponential phase cultures, and we now know that CIO expression is increased rapidly and up to 5-fold once the culture enters stationary phase. Therefore, we repeated these activity measurements on the strains used in this study, taking particular care to grow cultures to mid-exponential phase (well before CIO expression increases) and into stationary phase (where CIO has been induced). These data, determined as succinate-dependent CIO activity on whole cells, are reported in Table I. What is clear is that pLC2 and particularly pLC8 have a marked effect on CIO activity (up to a 2-fold increase) in wild-type and mutant backgrounds in exponential but not in stationary phase cultures. In contrast pLC1 had little or no effect on CIO activity in exponential phase cultures. No effects were observed with the pUCP18 control plasmid. Therefore, we conclude that either mutation or the presence of cioAB in trans at levels that lead to overexpression of CIO activity in growing cultures leads to a temperature-sensitive growth phenotype in P. aeruginosa.

As has been shown previously for the Ts growth phenotype of E. coli cytochrome bd mutants (19), the Ts defect of the cio mutant and pLC2/pLC8 containing strains of P. aeruginosa could be suppressed by thiol-reducing agents and catalase. The thiol-reducing agents l-cysteine, reduced glutathione, β-mercaptoethanesulfonic acid, and diithiothreitol and catalase suppressed the Ts phenotype. This was shown by dense halos of growth on agar plates around filter disks containing these compounds for the cio mutant and complemented mutant, when grown at 42 °C (data not shown).

cio Mutant and cioAB Plasmid-containing Strains Form Non-septate, Multinucleated Filaments—While looking at the Ts phenotype of cio mutant- and cioAB plasmid-containing strains, we observed clumps in cultures, the numbers of which increased as the growth temperature was raised. Microscopic examination of cultures following 4',6-diamidino-2-phenylindole staining indicated that both mutation and the presence of the cioAB genes on a multicopy plasmid led to the formation of non-septate, multinucleated filaments in a temperature-sensitive manner (Fig. 2). Wild-type cultures contained small rods at all growth temperatures (Fig. 2, A-C). However, the cio mutant formed occasional short filaments at 30 °C (Fig. 2F), but these become longer and more frequent as the growth temperature was raised to 37 °C. At 42 °C most cells in cio mutant cultures were filamentous and indeed fell to the bottom of a static culture vessel (Fig. 2H), as was also the case in pLC8 complemented mutant cultures. Introduction of the cioAB-containing plasmids pLC8 or pLC2 into the wild-type also induced filamentation in a temperature-dependent manner (data not shown), with filamentation most pronounced at 42 °C (Fig. 2E). However, as with the Ts phenotype, introduction of pLC1 into the wild-type had little effect on cell morphology and a comparatively minor effect in a cioAB mutant background (Fig. 2, D and I). No effect on cellular morphology was seen with pUCP18 control plasmids (data not shown). We conclude that strains mutated in cio or carrying the cioAB genes on a multicopy plasmid have a cell division defect that lies in one of the processes associated with septum formation. These strains also had a problem exiting stationary phase. If cultures were grown to stationary phase at 37 °C, subcultured into fresh medium, and incubated at 30 °C then the wild-type resumed exponential growth immediately, whereas the cioAB mutant or pLC2/pLC8-containing strains showed a significant lag phase, often of many hours, before resuming growth. Microscopic observation showed that during this lag period the filaments broke down, and exponential growth only commenced when the population was almost totally comprised of single cells (data not shown).

Mutation of cioAB Leads to Multiple Antibiotic Sensitivity—Given the pleiotropic effects of cioAB mutation on P. aeruginosa, we looked at its effect on a critical biological property of this bacterium; its efflux pump-mediated multiple antibiotic resistance (25). We found that the cio mutant and cioAB plasmid-containing strains had a multiple antibiotic-sensitive phenotype, which was temperature-dependent (Table II). Strains mutated in cioAB or with cioAB on multicopy plasmids were more sensitive to a range of antibiotics including chloramphenicol, β-lactams, quinolones, and macrolides at all growth temperatures. At 30 °C (data not shown) and 37 °C (Table II) there was a 2–8-fold decrease in the MIC for a range of different antibiotics. However, a much greater decrease in MIC for the same antibiotics was found in cultures grown at 42 °C. Antibiotic sensitivity was also found in wild-type/pLC8 but not in strains containing the control plasmid pUCP18 (data not shown). The antibiotic sensitivity of the strains carrying pLC2/pLC8 was greater than that of the cio mutant strain. The addition of exogenous catalase (~500 units) resulted in a 5–10-fold increase in the MIC of the cio mutant and PAO7701/pLC2 strains at 37 and 42 °C but no change in that of the wild-type (Table III). This suggests that damage caused by reactive oxygen species is a contributory factor to the multiple antibiotic-sensitive phenotype.

Protonmotive force-dependent multidrug efflux pumps, many with unusually broad specificity, play a major role in intrinsic antibiotic resistance (25). Four have been described in P. aeruginosa, of which one, the MexAB-OprM efflux system, which pumps out β-lactams, tetracycline, chloramphenicol, and quinolones, as well as trimethoprim and sulfame-
thoxazole, is the major contributor to the high intrinsic antibiotic resistance of *P. aeruginosa* (21, 26–29). The multiple antibiotic-sensitive phenotypes observed could result from damage to a multidrug efflux pump. Therefore, we determined the \( \text{H}_9004 \)-dependent accumulation of chloramphenicol in various strains as a function of temperature (Fig. 3). There was a progressive impairment of \( \text{H}_9004 \)-dependent chloramphenicol accumulation in the PAO7701 and PAO7701/pLC2 strains, as the growth temperature was increased (Fig. 3), which parallels the temperature-dependent changes in multiple antibiotic sensitivity demonstrated (Table II). PAO7701 and PAO7701/pLC2 grown at 42 °C immediately accumulated \(^{[3]}\text{H}\)-chloramphenicol to levels only observed in the wild-type following treatment with the protonophore carbonyl cyanide \( \text{m}- \)chlorophenylhydrazone. This is consistent with a complete loss of \( \text{H}_9004 \)-dependent chloramphenicol efflux in both the cio mutant and strains containing *cioAB* on a multicopy plasmid at this temperature.

**FIG. 2.** Fluorescence photomicrographs of 4′,6-diamidino-2-phenylindole-stained *P. aeruginosa* strains. Cultures were grown at 30 °C (A and F), 37 °C (B and G), and 42 °C (C–E and H–J) and stained as described under “Materials and Methods.” Wild-type strain PAO6049 (A–C), PAO6049/pLC1 (D), PAO6049/pLC8 (E), PAO7701 (F–H), PAO7701/pLC1 (I), and PAO7701/pLC8 (J) are shown.

**TABLE II**

**Susceptibility of *P. aeruginosa* to antimicrobial agents**

| Drugs          | MIC\( ^a \) for strain at 37 °C | MIC for strain at 42 °C |
|----------------|---------------------------------|------------------------|
|                | PAO6049 | PAO7701 | PAO7701/pLC2 | PAO6049 | PAO7701 | PAO7701/pLC2 |
| Chloramphenicol| 100     | 25      | 25          | 50      | 6.25    | 0.781       |
| Nalidixic acid | 100     | 25      | 25          | >100    | 25      | 25          |
| Tobramycin     | 3.125   | 0.781   | 0.390       | 1.562   | <0.195  | <0.097      |
| Gentamycin     | 6.25    | 0.781   | 0.781       | 1.562   | <0.195  | <0.097      |
| Norfloxacin    | 1.562   | 0.781   | 0.781       | 1.562   | <0.195  | <0.097      |
| Novobiocin     | 1000    | 500     | 500         | >1000   | 500     | 500         |
| Carbenicillin  | 50      | 12.5    | 25          | 25      | 3.125   | 12.5        |
| Fusidic acid   | 1000    | 500     | 500         | 100     | 25      | 12.5        |
| 2-2′-Dipyridyl | 50      | 25      | 25          | 25      | 3.125   | 1.562       |
| Erythromycin   | 1000    | 500     | 500         | 1000    | 125     | 125         |

\( ^a \) MIC values are reported as \( \mu \text{g/ml} \), except for 2-2′dipyridyl, which is in mg/ml.

**TABLE III**

**Chloramphenicol sensitivity is corrected by addition of catalase**

| Addition | MIC for chloramphenicol at 37 °C | Mic for chloramphenicol at 42 °C |
|----------|---------------------------------|---------------------------------|
|          | PAO6049 | PAO7701 | PAO7701/pLC2 | PAO6049 | PAO7701 | PAO7701/pLC2 |
| None     | 12.5    | 3.25    | 0.781        | 50      | 6.25    | 0.390       |
| Catalase | 12.5    | 6.25    | 6.25         | 50      | 12.5    | 1.562       |

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**Time (mins)**

**Fig. 3.** Accumulation of [3H]chloramphenicol by CIO-defective strains. Cultures were grown at 30 °C (A), 37 °C (B), and 42 °C (C) to mid-exponential phase, harvested, and resuspended in phosphate buffer/MgSO4/glucose. [3H]Chloramphenicol accumulation was determined as described under “Materials and Methods.” Dashed and solid lines indicate samples treated and untreated with carbonyl cyanide m-chlorophenyl-hydrazone, respectively. ● PAO6049; ▲ PAO7701; ● PAO7701/pLC2.

**cio Mutant and cioAB Plasmid-containing Strains Are Oxidatively Stress—**We investigated further the possibility that increased levels of reactive oxygen species might be a common cause of the diverse phenotypes observed. Consistent with this was the finding that the cio mutant and plasmid pLC8-containing strains were more sensitive to H2O2 than the wild-type (Fig. 4). Furthermore, we found these strains contained higher levels of oxidatively modified proteins (Fig. 5). Protein carbonyls can be used as a general measure of oxidative damage to proteins (30). At 37 and 42 °C protein carbonyl levels were higher in the cio mutant and in the strains PAO7701/pLC8 and PAO6049/pLC8 than in the wild-type, and this was most apparent at 42 °C (Fig. 5B). These data suggest that in strains mutated in cioAB or carrying plasmid-borne copies of the cioAB genes there is increased oxidative damage to proteins, particularly at higher temperatures.

**cio Mutant and cioAB Plasmid-containing Strains Have Reduced Catalase Activity—**Oxidative stress levels in a cell can increase as a consequence of increased rates of production or decreased rates of decomposition of reactive oxygen species. Therefore, we looked at hydrogen peroxide production rates and steady state concentrations in cells, as well as catalase activity. The rates of H2O2 production by cytoplasmic membrane fractions were similar in wild-type, mutant, and cioAB plasmid-containing strains (data not show). However, there were small differences in the steady state levels of H2O2 in cultures of these strains, but these changes were not significant at a p < 0.005 (data not shown). However, there was a statistically significant lower catalase activity in the mutant and cioAB plasmid-containing strains at all three growth temperatures (Fig. 6A). Furthermore, localization of catalase activity in stained native gels indicated that the catalase expression pattern was temperature-dependent and different among wild-type, mutant, and plasmid-containing strains (Fig. 6B). Two bands were visible for the wild-type growing at 37 and 42 °C (Fig. 6B, lanes 6 and 10), but only one band was visible for the cioAB mutant and the complemented mutant and wild-type/pLC8. In the wild-type the faster migrating band was temperature-induced, as it was absent from the wild-type grown at 30 °C, was only very faint, but reproducibly so, at 37 °C, and was most evident in 42 °C cultures (Fig. 6B). As these catalase bands were not paraquat-inducible neither can be assigned as the P. aeruginosa KatB (31) (data not shown). Therefore, the catalase bands observed must result from the major catalase in P. aeruginosa, KatA (32), probably the slower migrating band that is present at all temperatures, and KatC. KatC is a third catalase predicted to be encoded by the P. aeruginosa genome (4). We suggest it might be KatC whose levels are affected in the cio-defective strains, as the temperature-inducible catalase is barely detectable at 37 °C in the wild-type, a temperature at which KatA is expressed at significant levels (32).

**DISCUSSION**

The important finding of this study is that mutation of the cyanide-insensitive terminal oxidase of P. aeruginosa leads to a remarkable range of phenotypes. These include temperature sensitivity for growth, a phenotype that has been shown previously to be associated with mutation of genes required for the production of a functional cytochrome bd oxidase in E. coli (12–16, 19). However, the phenotypes elicited by mutation of the CIO in P. aeruginosa are significantly more broad ranging. These include defects in two critical biological properties of P. aeruginosa, its cell division cycle and multiple antibiotic resistance, and are brought about by the presence of multiple,
Strains were grown in LB medium to early stationary phase at 37 °C or 42 °C (B), and protein carbonyl levels were determined as described under “Materials and Methods.” Slot 1, wild-type PA06049; slot 2, PA06049/pLC8; slot 3, PA07701; slot 4, PA07701/pLC8. 7.5 µg of protein was loaded per slot.

The cloned cioAB genes on the plasmids pLC2 and pLC8 complement the cioAB mutant for its defect in CIO activity but also lead to overexpression of oxidase activity compared with the wild-type (5, 6) (Table I). Although there is clear evidence that the cioAB genes form an operon (5), the plasmids pLC2 and pLC8 did not complement the Ts, cell division, or multiple antibiotic-sensitive phenotypes. Introduction of these plasmids into the wild-type led to similar and often more severe phenotypic changes than in the cioAB mutant, which indicates that the phenotypes did not result from a polar effect of the mutation but rather depended on the presence of plasmid-borne copies of the cioAB. In contrast, strains containing the plasmid pLC1 showed modest (6) or insignificant changes in CIO activity compared with the wild-type (Table I) and were only slightly affected in growth at 42 °C and showed little of no filamentation (Fig. 2). Therefore, it is possible that the phenotypes result in part from the increased exponential phase CIO activity present in strains containing the plasmids pLC2 and pLC8 (Table I).

The temperature-dependent formation of non-septate, multinucleated filaments was indicative of a defect in cell division following chromosome replication and segregation but before septum formation (33). This may have resulted from the targeting by an unknown mechanism (see below) of a key component of the apparatus associated with septum formation (33). The cell division defect also explains why CIO-defective strains of P. aeruginosa have difficulty exiting stationary phase as exponential growth only commences once all of the filaments have broken down into single cells. A cell division defect may also explain the stationary phase exit phenotype of cytochrome bd mutants of E. coli (14).

P. aeruginosa is renowned for its intrinsically high levels of resistance to a wide range of antibiotics (25). A combination of the relatively low permeability of its cell envelope and the presence of multidrug efflux pumps, with a broad specificity for different classes of antimicrobial agents, explains this resistance (25). The cio mutant and cioAB plasmid-containing strains were markedly more sensitive to a range of antibiotics, a pattern consistent with a loss of activity of the constitutively expressed MexAB-OprM efflux pump of P. aeruginosa (21, 27, 34). The loss of uncoupler-dependent chloramphenicol uptake in CIO-defective strains as the growth temperature was increased was consistent with the impairment of or loss of efflux pump function. What is uncertain is whether this effect reflected a change in efflux pump expression, stability, or damage to pre-existing pumps components. Three further efflux pumps have been characterized in P. aeruginosa. These include MexCD-OprJ, MexEF-OprN, whose expression is de-repressed in nfxB and nfxC mutant backgrounds, respectively (35), and MexXY-OprM, which is induced in response to the presence of certain antibiotics (36). It will be interesting to determine whether CIO defects also lead to multiple antibiotic sensitivity in nfxB and nfxC strains.

So how do cio mutation and the presence of multiple copies of cioAB in trans on multicopy plasmids both lead to similar phenotypes, and is the same mechanism involved in each case? Overexpression of cioAB leads to a new spectral signal appearing in cytoplasmic membrane preparations, which may result from abhorrent home incorporation (5). This may result in the presence of non-functional forms of the oxidase in the membrane that disrupt electron transfer in vivo. Alternatively, an inappropriate complement of terminal oxidases, whether this be through the absence of an oxidase through mutation or through the presence of non-ideal levels, may interfere with optimal respiratory performance. One of the issues that is clear from this study is the need for P. aeruginosa, and perhaps other bacteria with similar oxidases, to carefully control the levels of the CIO, as inappropriate levels of expression can have significant consequences for the cell. Evidence from complementation studies suggests that the mini-D171 insertion used to construct PA07701 is in cioB. Therefore, a further possibility is that the phenotypes (and perhaps those of E. coli cytochrome bd mutants) might arise from truncated subunits or imbalances in the amounts (or function) of CioA or CioB.

A key question is how does a defect in the CIO lead to such a range of phenotypes, and do the CIO phenotypes result from a common mechanism or from multiple causes? As has been found for E. coli cytochrome bd mutants (14, 11, 19) the Ts phenotypes of mutated and cioAB plasmid-containing strains of P. aeruginosa can be suppressed by reducing agents and catalase. Furthermore, the antibiotic sensitivity of P. aeruginosa can also be partially restored in the presence of catalase. The increased sensitivity of the cio mutant and cioAB-contain-

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*G. R. Tavankar and H. D. Williams, unpublished data.*
ing plasmids to H₂O₂ also supported the idea that these strains are oxidatively stressed. Although we could not show statistically significant differences in the steady state H₂O₂ levels between strains, the protein carbonyl levels were higher at each of the three temperatures tested in the CIO-defective strains, indicating increased oxidative protein damage (30). So oxidative modification to key proteins with their likely loss of function may explain some of the phenotypes observed. The absence of a specific catalase isoenzyme and subsequent less efficient removal of H₂O₂ is consistent with the decreased H₂O₂ resistance of CIO-defective strains and the increased oxidative protein damage observed. The temperature dependence of this catalase activity also may explain the Ts nature of the phenotypes. However, two outstanding questions are the identity of this catalase and how its production is affected in CIO mutants. Although oxidative damage to proteins may be an important factor it is likely to be a secondary consequence of the loss of catalase activity. An understanding of the mechanism behind the failure of CIO-defective strains to produce a functional catalase will provide a more complete and all-encompassing explanation of the molecular basis of the phenotypic properties of CIO-defective strains of P. aeruginosa.

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