Regulation of expression of the cyanide-insensitive terminal oxidase in *Pseudomonas aeruginosa*

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The regulation of the cyanide-insensitive oxidase (CIO) in *Pseudomonas aeruginosa*, a bacterium that can synthesize HCN, is reported. The expression of a cioA–lacZ transcriptional fusion, CioA protein levels and CIO activity were low in exponential phase but induced about fivefold upon entry into stationary phase. Varying the O₂ transfer coefficient from 11·5 h⁻¹ to 87·4 h⁻¹ had no effect on CIO expression and no correlation was observed between CIO induction and the dissolved O₂ levels in the growth medium. However, a mutant deleted for the O₂-sensitive transcriptional regulator ANR derepressed CIO expression in an O₂-sensitive manner, with the highest induction occurring under low-O₂ conditions. Therefore, CIO expression can respond to a signal generated by low O₂ levels, but this response is normally kept in check by ANR repression. ANR may play an important role in preventing overexpression of the CIO in relation to other terminal oxidases. A component present in spent culture medium was able to induce CIO expression. However, experiments with purified N-butanoxy-L-homoserine lactone or N-(3-oxododecanoyl)homoserine lactone ruled out a role for these quorum-sensing molecules in the control of CIO expression. Cyanide was a potent inducer of the CIO at physiologically relevant concentrations and experiments using spent culture medium from a ∆hcnB mutant, which is unable to synthesize cyanide, showed that cyanide was the inducing factor present in *P. aeruginosa* spent culture medium. However, the finding that in a ∆hcnB mutant cioA–lacZ expression was induced normally upon entry into stationary phase indicated that cyanide was not the endogenous inducer of the terminal oxidase. The authors suggest that the failure of O₂ to have an effect on CIO expression in the wild-type can be explained either by the requirement for an additional, stationary-phase-specific inducing signal or by the loss of an exponential-phase-specific repressing signal.

INTRODUCTION

*Pseudomonas aeruginosa* is an opportunistic pathogen that causes a variety of nosocomial infections, including pneumonia, urinary tract infections, surgical wound infections, and bloodstream infections (for a review see Deretic, 2000). It causes life-threatening illness in patients with cystic fibrosis. Initially, *P. aeruginosa* colonizes the airways with other pathogens such as *Haemophilus influenzae* and *Staphylococcus aureus*. However, in most of these patients chronic lung disease develops in which the bacterial population consists almost exclusively of *P. aeruginosa* in the form of biofilms (Govan & Deretic, 1996). *P. aeruginosa* is a facultative anaerobe that preferentially obtains its energy via aerobic respiration, but it is well adapted to conditions of limited O₂ supply (Palleroni, 1984; Davies et al., 1984; Zannoni, 1989).

Most facultative anaerobes contain multiple energy-generating pathways, the synthesis of which is often controlled by overlapping regulatory circuits. By controlling the expression of particular electron-transfer components, especially its cytochrome oxidases, the bacterium constructs the most appropriate electron-transfer chain for the prevailing environmental conditions (Poole & Cook, 2000; Richardson, 2000). Analysis of the genome sequence of *P. aeruginosa* indicates that it has the potential to make a complex, highly branched aerobic respiratory chain comprising up to five terminal oxidases (Stover et al., 2000; M. Cooper & H. D. Williams, unpublished; Fig. 1). Of four putative terminal oxidases belonging to the haem–copper oxidase superfamily, three are predicted to be cytochrome c oxidases and one a quinol oxidase (Fig. 1). The exception is the cyanide-insensitive oxidase (CIO), encoded by the cioAB operon, which is homologous to the cytochrome bd oxidase as a terminal electron acceptor and in the absence of nitrate it is able to ferment arginine, generating ATP by substrate-level phosphorylation (Palleroni, 1984; Davies et al., 1989; Van der Wauven et al., 1984; Zannoni, 1989).
The bacterial strains used in this study are listed in Table 1. *P. aeruginosa* was grown in LB medium at 30 °C. We found it to be important to use cultures that were well adapted to exponential phase in order to get reproducible gene expression data, particularly from the early exponential phase of growth. Therefore, an overnight culture was diluted 1:100 into fresh medium and grown to mid-exponential phase, subcultured, and again grown to mid-exponential phase before being used as the inoculum for the experimental flask. To vary the O₂ supply to cultures, they were grown at three oxygen-transfer coefficient (k₁a) values (Pirt, 1975): high (87.4 h⁻¹), medium (27.8 h⁻¹) and low (11.5 h⁻¹). This was achieved by using identical 250 ml flasks, shaken at 200 r.p.m., but altering the medium volume. The k₁a values for medium volumes of 25 ml (high), 75 ml (medium), and 150 ml (low) were estimated by the sulphite oxidation method (Pirt, 1975; Ruchti et al., 1985; Gil et al., 1992; D’Mello et al., 1994).
Anaerobic cultures were grown in 50 ml LB medium with 0·2 % (w/v) nitrate in 50 ml Falcon tubes and incubated at 30 °C, without shaking, in a GasPak anaerobic jar system (Beckton Dickinson). \textit{P. aeruginosa} was cultured twice anaerobically into exponential phase before inoculation of the experimental culture.

### Determination of percentage \( \mathrm{O}_2 \) saturation

An MI-730 dip-type oxygen microelectrode and OM-4 \( \mathrm{O}_2 \) meter (Microelectrodes Inc.) were used for dissolved \( \mathrm{O}_2 \) tension measurements. So that they could accept the electrode, 250 ml flasks were modified by addition of a glass inlet tube. The inlet tube was continuous with the main flask, and positioned 5 mm from the bottom of the flask to ensure that the probe was submerged at all times by the lowest volume of medium (25 ml). An O-ring was positioned at the entry point to the flask to hold the tip of the electrode and to prevent culture fluid entering the inlet tube. The electrode was further held in place by a sealed cap at the far end of the inlet tube.

### Design of anti-CioA antibody and Western blotting

A region of the CioA subunit of the CIO enzyme was selected for production of anti-CIO antibodies. The region selected had to be susceptible to antibody binding, and therefore be on the outer surface of the protein. The cytochrome \( bd \) oxidase of \textit{E. coli} has a large periplasmic loop known as the Q-loop, within which a stretch of 11 amino acids forms part of the quinol-binding site (Dueweke & Gennis, 1990). These 11 amino acids were mapped as the epitope for monoclonal antibodies. The Q-loop of \textit{P. aeruginosa} is more than 60 amino acids shorter than that of \textit{E. coli}, and of the 11 amino acids mapped as part of the quinol-binding site, 5 are completely conserved and a further 3 are conservatively substituted (Cunningham et al., 1997). To check whether this region of the CioA protein was likely to be antigenic, the Protein program of the DNASTAR software suite was used to calculate the Jameson–Wolf antigenic index for regions within the CioA protein. This analysis demonstrated that the 11 amino acid epitope to CydA was not the best site for antibody design in the CioA protein. A different region within the Q-loop was selected with a more suitable antigenic index and the chosen epitope (KIAAMEGHWDN) was then synthesized and used for the production and affinity purification of rabbit anti-CioA antibodies by Research Genetics Inc. The antibody was used in Western and slot blotting using standard procedures.

### DNA manipulations

General DNA manipulations were carried out as described by Sambrook \textit{et al.} (1989).

### Construction of a \( \text{cioA}--\text{lacZ} \) transcriptional gene fusion

Using the \text{cioAB} sequence data (Cunningham \textit{et al.}, 1997), primers were designed flanking the promoter region of the \text{cioAB} genes. The forward primer (pcioF, CGGCCAGCGACTTGTATTTC) was located upstream of a convenient \text{PstI} site; the reverse primer (pcioR, CTAGGCAATGCCCCATGCGAAGTTGACC) was located downstream of the promoter region, and had a \text{SphI} site incorporated onto the 5’ end. PCR was performed using pLC2, a plasmid containing the entire \text{cioAB} region, including the promoter region (Cunningham & Williams, 1995; Cunningham \textit{et al.}, 1997), as the template. The amplified promoter segment of 995 bp was cloned into the pGEMT-easy vector (Promega) and removed as a \text{PstI–SphI} fragment. This fragment was then cloned into a \text{PstI}–\text{SphI}-restricted \text{pMP220} vector (Spaink \textit{et al.}, 1987), placing it upstream of a promoterless \text{lacZ} gene, to construct pMC10. pMC10 was then transformed into \textit{E. coli} XL-1 Blue, reisolated and sequenced. pMC10 was introduced into \textit{P. aeruginosa} strains by triparental conjugation (Rothmel \textit{et al.}, 1991). \( \beta \)-Galactosidase assays were carried out according to Miller (1972) and all experiments were repeated at least three times. Representative experiments are shown and all data points are ± SD.

### CIO activity measurements

Succinate-dependent \( \mathrm{O}_2 \) uptake was determined in whole cells in the presence of 1 mM KCN as described previously (Cunningham & Williams, 1995).

### RESULTS

#### The CIO is induced upon entry into stationary phase

The expression of the CIO was measured in three ways: by using a \text{cioA}--\text{lacZ} transcriptional fusion (Fig. 2a–c); by Western/slot blotting using a polyclonal antiserum raised to a synthetic peptide sequence from the major periplasmic (Q-) loop of CioA (Fig. 2d); and by determining CIO activity as succinate-dependent \( \mathrm{O}_2 \) uptake in whole cells in the presence of 1 mM KCN (Fig. 2e). Each of these approaches indicated that CIO expression was lowest in exponential phase and increased rapidly to a maximum in stationary phase. \text{cioA}–\text{lacZ} expression increased immediately as growth stopped and the culture entered stationary phase, reaching a maximum of approximately five times the exponential phase levels about 2·5 h into stationary phase. This change was paralleled by a similar fivefold increase in CIO activity and a clear increase in CioA protein levels.

#### The role of oxygen in CIO expression

Oxygen is a major factor in the regulation of terminal oxidases in many bacteria (Richardson, 2000). To investigate its role in the regulation of the CIO, we followed CIO expression throughout the growth cycle in cultures grown with different \( \mathrm{O}_2 \) transfer coefficients (\( k_a \)) and at the same time monitored \( \mathrm{O}_2 \) levels directly in the culture flasks using an \( \mathrm{O}_2 \) microelectrode. Firstly we determined the growth properties and the percentage \( \mathrm{O}_2 \) saturation during growth at three different \( k_a \) values (Fig. 3a). Decreasing the \( k_a \)
**Fig. 2.** Effect of growth phase and varying the $k_La$ on CIO expression. (a, b, c) *P. aeruginosa* was grown in LB medium at high ($k_La = 87.4 \text{ h}^{-1}$), medium ($k_La = 27.8 \text{ h}^{-1}$) and low ($k_La = 11.5 \text{ h}^{-1}$) $k_La$ values. Growth as OD$_{600}$ (filled symbols) and $\beta$-galactosidase levels (open symbols) are shown for the control vector pMP220 (circles), or pMC10 ($cioA$–$lacZ$ transcriptional fusion, squares). (d) Protein (10 mg) from exponential-phase (Exp) or stationary-phase (SP) cells grown at high, medium or low $k_La$ as indicated was used in a slot blot assay with anti-CioA antibody. (e) CIO activity measurements [nmol O$_2$ min$^{-1}$ (mg protein)$^{-1}$] made on cells from exponential- or stationary-phase cultures grown at the indicated $k_La$ values.

**Fig. 3.** Effect of $k_La$ on growth and percentage O$_2$ saturation of *P. aeruginosa* cultures. (a) Wild-type PAO1; (b) PAO6261 $\Delta$anr. The percentage O$_2$ saturation (filled symbols) was measured throughout the growth of *P. aeruginosa* in cultures in LB medium at different $k_La$ values: high (87.4 h$^{-1}$, circles), medium (27.8 h$^{-1}$, triangles), low (11.5 h$^{-1}$, squares). Growth was measured as OD$_{600}$ (open symbols).
had no significant effect on the growth rate but it did reduce the final culture optical density (Fig. 3a). At high \( k_La \) the \( O_2 \) levels reached a minimum of approximately 4% \( O_2 \) saturation just as the culture was about to enter stationary phase. At medium \( k_La \) the \( O_2 \) levels reached zero after 5 h of growth just as the culture was entering stationary phase, while at low \( k_La \) the percentage dissolved \( O_2 \) saturation reached zero after 2.5 h, when the culture was in mid-exponential phase.

Varying the \( k_La \) resulted in a modest increase in the stationary-phase \( \beta \)-galactosidase activity from 1400 units at high \( k_La \) to 1800 units at low \( k_La \) but had no major effect on CIO activity or CioA protein levels in exponential or stationary phase (Fig. 2). Furthermore, the point in the growth curve at which \( cioA-lacZ \) expression was induced was the same irrespective of when the dissolved \( O_2 \) concentrations reached a minimum (Figs 2 and 3). Therefore, based on these data we conclude that under the conditions used here varying \( O_2 \) has no effect on CIO expression.

\( P. aeruginosa \) can grow under anaerobic conditions using nitrate respiration. During anaerobic growth with nitrate as the terminal electron acceptor \( cioA-lacZ \) expression levels were constant at about 300 units \( \beta \)-galactosidase throughout the growth curve, which is similar to the basal level seen in exponential phase during aerobic growth. There was a similar, low level of CioA detected by Western blotting throughout growth (data not shown).

The oxygen-sensitive transcriptional regulator ANR is a repressor of the CIO

We previously found that mutation of the \( O_2 \)-sensitive transcriptional regulator ANR led to a marked increase in CIO activity (Ray & Williams, 1997). Therefore, despite the fact that changing \( O_2 \) availability by varying the \( k_La \) did not alter CIO expression (Fig. 2), we looked again at the effect of an \( anr \) mutation on CIO expression. The effect of \( k_La \) on growth and the percentage dissolved \( O_2 \) saturation of the \( anr \) mutant culture is shown in Fig. 3(b); the growth results are similar to those for the wild-type.

In the \( \Delta anr \) mutant \( cioA-lacZ \) expression was derepressed upon entry into stationary phase (Fig. 4). The extent of this derepression was dependent upon the \( k_La \) and was most marked at low \( k_La \), a condition under which ANR is most active (Galimand et al., 1991; Sawers, 1991; Zimmerman et al., 1991). In addition, the timing of \( cioA-lacZ \) expression was advanced as the \( k_La \) was lowered (Fig. 4c). This change in \( cioA \) promoter activity was reflected in increased CioA levels detected by Western blotting and CIO activity assays (Fig. 4). \( P. aeruginosa \) has a second FNR homologue known as DNR, which has a role in the regulation of anaerobic respiratory pathways involved in denitrification (Arai et al., 1995). However, mutation of \( dnr \) had no effect on \( cioA-lacZ \) expression or CioA levels, irrespective of the \( k_La \) (data not shown), ruling out a role for DNR in CIO regulation.
What is the stationary-phase inducing factor?

The data above indicate that O₂ can generate a signal that leads to induction of the CIO (in the anr mutant), but suggest that it may not be responsible for the stationary-phase induction observed in wild-type cultures. Consequently, we investigated three alternative explanations for the stationary-phase induction of the CIO, as follows.

Induction by the stationary-phase sigma factor RpoS.

The sigma factor RpoS is known to have a role in regulating the expression of stationary-phase genes in a wide range of bacteria, including *P. aeruginosa* (Jorgensen et al., 1999; Latifi et al., 1996; Suh et al., 1999; Tanaka & Takahashi, 1994; You et al., 1998). However, an rpoS mutation had no effect on *ciao–lacZ* expression or CioA levels during growth at all three k₅a values, ruling out a role for this sigma factor in the regulation of CIO (data not shown).

Quorum sensing. Quorum sensing via the *lasRI* and *rhlRI* systems is well established as regulating a range of stationary-phase phenomena in *P. aeruginosa* (Fuqua & Greenberg, 1998; Withers et al., 2001; Iuchi & Lin, 1993; Swift et al., 2001). We investigated whether quorum sensing was responsible for stationary-phase induction of the CIO, by looking at the effect of adding spent medium from stationary-phase cultures on *ciao–lacZ* expression (Fig. 5). Addition of spent culture medium increased *ciao–lacZ* expression in a dose-dependent manner, with a more marked effect on exponential-phase cultures (Fig. 5a). Similar results were obtained at each of the three k₅a values tested (data not shown). Fig. 5(b) shows the kinetics of *ciao–lacZ* induction upon addition of 60% (v/v) spent culture medium to a culture. A sharp increase in CIO promoter activity is seen in exponential phase, when *cioAB* expression is usually at its lowest. Expression began to fall after mid-exponential phase and continued to fall after entry into stationary phase, when *cioAB* expression would usually be rising. The pattern was the same at all three k₅a values tested (data not shown). However, the factor inducing the CIO was not one of the two major *N*-acylhomoserine lactones synthesized by *P. aeruginosa*, as addition of neither *N*-butanoyl-*L*-homoserine lactone (BHL) nor *N*-(3-oxododecanoyl) homoserine lactone (OdDHL), nor of both added together, at a concentration of 5 µM had any effect on the expression of the CIO (data not shown). A dose–response experiment using concentrations of OdDHL and BHL from 5 to 50 µM also did not elicit activation of *cioA–lacZ* expression (data not shown).

Cyanide. The CIO is fully active in the presence of 1 mM KCN while the alternative respiratory pathways, terminated by haem–copper oxidases, are inhibited at this concentration; indeed they have an IC₅₀ of about 50 µM (Cunningham et al., 1997; Matsushita et al., 1983). It is known that HCN levels peak in stationary phase and under the growth conditions used here reach 200–300 µM (data not shown) and so an important question is whether *cioAB* and *hcnABC* expression are coordinated in some way. Therefore, we investigated the possibility that cyanide could be the stationary-phase inducing factor. When cyanide was added to exponential-phase cultures CIO expression increased markedly in a dose-dependent manner (Fig. 6a). Fig. 6(b) shows the effect of cyanide addition on expression during growth at high k₅a; similar data were obtained at other k₅a values tested (data not shown). Cyanide had a dramatic effect on expression, as expression was elevated up to 10-fold compared to the control culture and was maintained at a high level until late exponential phase, when it started to fall back to levels similar to the wild-type. Addition of cyanide had little effect on expression when added to stationary-phase cultures, in which the CIO is already maximally expressed. Cyanide does not act by disrupting the function of ANR, as cyanide was still able to induce CIO expression in an anr mutant (data not shown).
To test whether cyanide is the endogenous inducing factor for CIO expression we made use of a ΔhcncB mutant, PAO6344. PAO6344 produces no detectable levels of cyanide (unpublished results). Firstly, we showed that cyanide is an extracellular inducing factor, as spent culture medium from PAO6344 did not induce CIO expression to anything like the same level as spent medium from the wild-type (Fig. 7). This indicates that HCN is the major extracellular inducing factor and explains the quorum-sensing-type effect described above. However, it is noticeable that the kinetics of cioA–lacZ induction is advanced with spent medium from the ΔhcncB mutant and shows a modest increase in final β-galactosidase activity in stationary phase. So there may be an additional factor in the growth medium that can induce the CIO, but cyanide is the major effector. However, if cyanide is the endogenous stationary-phase inducing factor then CIO induction should not occur in the ΔhcncB mutant. However, cioA–lacZ expression and CioA levels were almost identical in wild-type and PAO6344 (data not shown), indicating that while HCN can induce the CIO it is not the endogenous inducing signal during entry into stationary phase.

**DISCUSSION**

Most facultative anaerobes contain multiple energy-generating pathways, the synthesis of which is often controlled by overlapping regulatory circuits. By controlling the expression of particular electron-transfer components the bacterium is able to construct the most appropriate electron-transfer chain for the prevailing environmental conditions (Poole & Cook, 2000; Richardson, 2000; Iuchi & Lin, 1993). While there are some studies of the regulation of anaerobic respiration in *P. aeruginosa* (Arai et al., 1994, 1995, 1997; Ye et al., 1995), the environmental and genetic factors that regulate its aerobic electron-transport chains have not been well studied. Moreover, as *P. aeruginosa* has the genetic potential to produce up to five terminal oxidases for use under aerobic conditions, in principle it has enormous flexibility in the choice of electron-transfer routes to *O₂*. However, there is a paucity of information on the function of the electron-transfer chains in this bacterium and so we have initiated a study of the regulation of the CIO of *P. aeruginosa*.

The CIO was growth-phase regulated as there was a marked decrease in β-galactosidase activity in the ΔhcncB mutant (data not shown), indicating that while HCN can induce the CIO it is not the endogenous inducing signal during entry into stationary phase.
increase in cioA–lacZ expression, CIO activity and CioA protein levels in stationary phase and the kinetics of cioA–lacZ expression indicated that this increase in expression started once growth had stopped. One explanation for this is that as the population density increases, oxygen levels are depleted and this generates a signal for CIO induction. We investigated the effect of O2 on growth by varying the rate of O2 supply and measuring the percentage O2 saturation in the culture during growth. This approach has allowed us to distinguish between stationary-phase and O2 effects on gene expression. Changing the rate of O2 supply had a clear physiological effect on the bacteria as seen by the reduced growth yield as the $k_1a$ decreased. If O2 regulates the CIO then a correlation between $k_1a$, the point at which O2 levels reached a minimum and the point of CIO induction might be expected; there was none.

The transcriptional regulator ANR, a homologue of FNR in E. coli, controls denitrification, arginine deiminase activity and cyanide production in P. aeruginosa (Galimand et al., 1991; Sawers, 1991; Zimmerman et al., 1991). Another CRP/FNR-related regulator, DNR, has also been shown to be necessary for denitrification, and transcription of the dnr gene is under the control of ANR (Arai et al., 1995, 1997). ANR controls expression of genes in response to intracellular O2 levels, whereas DNR is thought to respond to N-oxides (Hasegawa et al., 1998). The ANR protein is activated under low O2 levels and subsequently activates or represses genes with specific promoter sequences (ANR boxes) (Galimand et al., 1991; Sawers, 1991; Zimmerman et al., 1991). The cioAB promoter sequence contains two putative ANR boxes (Cunningham et al., 1997). Previously, we found that an anr mutant had increased CIO activity (Ray & Williams, 1997). Here we have shown that stationary-phase cioA–lacZ expression and CioA levels increase markedly in the anr mutant in an O2-dependent manner, being greatest at low $k_1a$. This is consistent with ANR repressing the expression of the cioAB operon in response to the low O2 levels encountered in stationary phase. Importantly, it indicates that CIO expression can respond to a signal generated by low O2 levels. So ANR may play an important role in preventing overexpression of the CIO in relation to other terminal oxidases. Indeed, the presence of the cioAB genes on a multicopy plasmid can lead to up to a fourfold increase in CIO activity with all the electron flux going to O2 via the CIO during NADH-dependent O2 uptake, even though significant cytochrome c oxidase activity is present in the cytoplasmic membranes (Cunningham & Williams, 1995; Cunningham et al., 1997).

The alternative sigma factor, RpoS, which has an established role in regulating stationary-phase phenomena (Jorgensen et al., 1999; Latifi et al., 1996; Suh et al., 1999; Tanaka & Takahashi, 1994; You et al., 1998), did not have a role in stationary-phase induction of the CIO. Initial data showing an effect of a spent medium component in activating CIO expression suggested a quorum-sensing-type control mechanism regulating the CIO. However, further experiments indicated that the extracellular inducing factor was cyanide.

We were interested to see whether CIO expression and HCN synthesis were coordinately regulated. Certainly both are expressed maximally in stationary phase and the synthesis of HCN is known to be dependent on the O2 concentration (Blumer & Haas, 2000; Castric, 1994; Castric et al., 1979). Furthermore, ANR regulates them in opposite ways, activating HCN synthesis but repressing CIO expression. However, we found that HCN can induce expression of the CIO at physiologically relevant concentrations. It is interesting that exogenous HCN is much less effective at inducing expression in stationary phase when the CIO is maximally expressed and, as a highly active component of the respiratory chain, is able to maintain electron flux to O2. This is consistent with cyanide inhibition of electron transport generating a signal for CIO induction. Evidence in support of this mechanism comes from work that demonstrated increased CIO activity in a ccmH mutant, which is defective in cytochrome c biogenesis and does not have an active cytochrome c oxidase terminated pathway (Ray & Williams, 1996). Such a mechanism would also be consistent with the effect of low O2 on CIO expression in a Δanr background. Otten et al. (2001) suggested that reduction of flux through the cytochrome c oxidase pathways enhanced activity and expression of the cytochrome ba3 quinol oxidase of Paracoccus denitrificans. They proposed that this effect was due to increased reduction of the quinone pool and that FnrP may have a role in sensing redox state as well as O2, enhancing expression of cytochrome ba3 when the quinone pool is highly reduced (Otten et al., 2001). The E. coli terminal oxidases are regulated in response to oxygen by the ArcAB two-component signal-transduction system, in which ArcB senses the redox state of the quinone pool (Georgellis et al., 2001). However, in Rhodobacter sphaeroides a two-component regulatory system PrrAB is proposed to regulate electron-transfer components by sensing the redox flux through the cytochrome c oxidizing pathway, specifically through a component of the cytochrome cbb3 (Oh & Kaplan, 2000, 2001). Very interesting, in this context, is the recent report of P. aeruginosa RoxR, a response regulator that is related to PrrA, with a role in regulating cioAB (Comolli & Donohue, 2002). The authors showed that mutation of roxR leads to the loss of cyanide and azide resistance in P. aeruginosa PAK, and provided evidence to support the idea that this resulted from CIO deficiency. However, the roxR mutant still showed significant cyanide-dependent induction of CIO activity and cioA–lacZ expression, indicating that there are other regulators still to be identified, with roles in the cyanide induction of CIO expression. In this paper, we have clearly shown that CIO is not induced by endogenously generated cyanide. It is important to note that Comolli & Donohue (2002) performed their experiments on exponential-phase cultures, that is, conditions under which the CIO expression is at a minimum and during which the endogenous stationary-phase inducing signal either is absent or has its
effects repressed. So, while an attractive model, it is premature to suggest that RoxR is responsible for the stationary-phase induction of CIO. Clearly it will be important to determine the growth-phase dependence of CIO induction in a rexR mutant.

An intriguing model is that HCN synthesis is switched on by ANR as the O$_2$ concentration drops and the cyanide inhibits electron transport by haem–copper oxidases, generating a signal that stimulates the induction of the CIO, allowing respiration to continue, with RoxR acting as the transcriptional regulator. However, HC$_N$ is not the stationary-phase inducing signal in vivo as the CIO was induced normally in a $\Delta$hcnB mutant that does not make cyanide. So does cyanide induction have any physiological relevance? Well, in a natural environment if an actively growing P. aeruginosa cell encounters other bacteria making cyanide then it could induce the CIO to protect itself. The identity of the endogenous inducing signal for stationary-phase induction of the CIO is unknown. However it could be a signal, such as the catabolic reduction charge, that reflects a change in the cell’s metabolic status following entry into stationary phase and which would affect both electron flux and the redox state of electron-transfer components. Does O$_2$ have a physiologically relevant role in the regulation of the CIO? How do we explain the apparent ability of low O$_2$ to generate an inducing signal, as evidenced by CIO expression patterns in a $\Delta$anr mutant, but the failure of changing the rate of O$_2$ supply to affect CIO regulation in the wild-type? The point in the growth curve at which stationary-phase induction of the CIO takes place is in all cases after O$_2$ levels have reached a minimum. So O$_2$ could be an inducing signal that is working in combination either with another stationary-phase induction signal or with an exponential-phase repressing signal. We favour the latter, as we have recently found that an increase in exponential-phase CIO activity, as a result of the cioAB genes being present on a multicopy plasmid, leads to a number of detrimental effects on the growth and physiology of P. aeruginosa (Tavankar et al., 2003). This suggests a requirement for tight regulation of the CIO in exponential-phase cultures. Therefore, in this model stationary-phase induction would result from a combination of the generation of an inducing signal by low O$_2$ levels and the loss of a repressing signal as the culture ceases exponential growth and enters stationary phase.

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REFERENCES


